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IDENTIFICACIÓN DE PRESENCIA O AUSENCIA DE GENES DE RESISTENCIA BACTERIANA A LOS ANTIBIÓTICOS (ARGs) EN MATERIA FECAL DE COYOTES (*Canis latrans*) DE DOS ÁREAS DE CONSERVACIÓN (GUANACASTE Y VALLE CENTRAL) EN COSTA RICA.

Sustentante

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Tesis sometida a consideración del Tribunal Examinador del Posgrado en Medicina de la Conservación, para optar al grado de Magister Scientiae en Medicina de la Conservación, énfasis Salud Ecosistémica.

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Resumen

La resistencia bacteriana a los antimicrobianos (RAM) se considera actualmente una enfermedad emergente que tiene un gran impacto en la salud pública a nivel mundial. Los antibióticos, hacen parte de este grupo de antimicrobianos, en donde las bacterias se vuelven resistentes a estos fármacos por adquirir genes de resistencia a los antibióticos (ARGs). Este proceso se perpetúa debido al bajo costo energético que tiene la adquisición de ARGs y por presiones antropogénicas y ambientales tales como el uso excesivo e indebido de los antibióticos, la insuficiente farmacovigilancia, el inadecuado manejo de desechos farmacéuticos y ambientales, como la amplia dispersión de ARGs por aire, suelo, agua y en la cadena alimenticia, que han exacerbado la capacidad de las bacterias para desarrollar resistencia.

Costa Rica, se ha vuelto un país líder en el monitoreo e investigación de la resistencia a los antibióticos, con un trabajo interdisciplinario que posiciona al país como referente en la investigación Centroamericana. Desde el componente ambiental, se han realizado investigaciones en diversos hábitats y entornos (cautiverio, sitios de producción acuícola y zonas de reserva) así como en diversidad especies: *Alouatta palliata, Ateles geoffrogyi, Saimiri Oerstedii, Procyon lotor, Panthera onca, Puma concolor, Columba livia, Oreochromis niloticus, Tapirus bairdii y Lontra longicaudis* (Angulo et al., 2023; Baldi et al., 2019; Blanco-Peña et al., 2024; Blanco-Peña et al., 2017; Gamboa et al., 2004; Oviedo-Bolaños et al., 2021; Rodríguez-Rodríguez et al., 2007; Rojas Jiménez, 2018; Rojas-Jiménez et al., 2019, 2022).

Esta investigación en particular se centra en el coyote (*Canis latrans*), un meso depredador con gran plasticidad y desplazamiento territorial, que lo convierte en un bioindicador de zonas buffer entre ambientes urbanos y rurales. Destacándose como una especie centinela crucial para esta interfaz, de gran importancia en el monitoreo, dado que las bacterias ambientales son más prevalentes y potenciales reservorios de ARGs. Por ello, este estudio se enfocó en caracterizar la presencia de ARGs asociados a cinco clases de antibióticos utilizados comúnmente en medicina veterinaria, humana y agricultura: tetraciclinas (*tet*W, *tet*Q, y *tet*Y), sulfonamidas (*sul*I y *sulI*I), fenicoles (*catA*I y *catA*II), quinolonas (*qnr*S) y

betalactámicos (*bla*$_{TEM}$). La detección se realizó mediante PCR punto final (PCR), PCR en tiempo real (qPCR) y secuenciación de Sanger en muestras colectadas a través de un método indirecto (heces) entre marzo y agosto de 2022, en las áreas de conservación de Guanacaste y el Valle Central.

Como resultado se determinó el estado del arte sobre la resistencia a antibióticos en las heces de los coyotes presentes en las dos zonas de conservación. Destacando la presencia significativa de ARGs y microbiomas multirresistentes en la población de coyotes muestreada. Gracias al análisis de datos a través de sistemas de georreferencia se evidenciaron asociaciones entre algunos genes y características geográficas. Información que permitió brindar recomendaciones puntales para la construcción de estrategias de mitigación a la resistencia a los antibióticos, específicamente en las clases de antibióticos evaluados en las zonas muestreadas.

Adicionalmente, este estudio proporciona los primeros datos genéticos mitocondriales del Gen D-loop en Costa Rica para las especies *C. latrans* y zorro gris (*Urocyon cinereoargenteus*). Permitiendo analizar preliminarmente la conectividad poblacional de los coyotes muestreados. Aportando datos base para futuros estudios sobre la salud poblacional de esta especie.

Abstract

Bacterial antimicrobial resistance (AMR) is currently considered an emerging disease that has a major impact on public health worldwide. Bacteria become resistant to antibiotics, which belong to the group of antimicrobials, by acquiring antibiotic resistance genes (ARGs). This process is perpetuated by the low energy cost of acquiring ARGs and by anthropogenic and environmental pressures. Among these factors we can find excessive and improper use of antibiotics, insufficient pharmacovigilance, inadequate management of pharmaceutical and environmental waste. All these factors cause the wide dispersion of ARGs through air, soil, water and in the food chain, and have exacerbated the ability of bacteria to develop resistance.

Costa Rica has become a leader in the monitoring and research of antibiotic resistance, with interdisciplinary work that positions the country as a reference in Central American research. From the environmental component, research has been carried out in different habitats and environments (captivity, aquaculture production sites and reserve zones) as well as in diverse species: *Alouatta palliata, Ateles geoffrogyi, Saimiri Oerstedii, Procyon lotor, Panthera onca, Puma concolor, Columba livia, Oreochromis niloticus, Tapirus bairdii and Lontra longicaudis* (Angulo et al., 2023; Baldi et al., 2019; Blanco-Peña et al., 2024; Blanco-Peña et al., 2017; Gamboa et al., 2004; Oviedo-Bolaños et al., 2021; Rodríguez-Rodríguez et al., 2007; Rojas Jiménez, 2018; Rojas-Jiménez et al., 2019, 2022).

This particular research focuses on the coyote (*Canis latrans*), a mesopredator with great plasticity and territorial displacement, which makes it a bioindicator of buffer zones between urban and rural environments. This condition makes the coyote a key sentinel species for this interface that may be of great importance in monitoring disease dynamics in its ecological niche where environmental bacteria are more prevalent and potential reservoirs of ARGs. Therefore, this study focused on characterizing the presence of ARGs associated with five families of antibiotics commonly used in veterinary, human and agricultural medicine: tetracyclines (*tet*W, *tet*Q, and *tet*Y), sulfonamides (*sul*I and *sul*II), phenicols (*cat*AI and *cat*AII), quinolones (*qnr*S) and beta-lactams (bla_{TEM}). Gene detection was performed by end-point PCR (PCR), real-time PCR (qPCR) and Sanger sequencing in samples collected

through an indirect method (feces) between March and August 2022, in the conservation areas of Guanacaste and the Central Valley.

Consequently, the current state of the art regarding antibiotic resistance in the feces of coyotes present in the two conservation areas was established. The considerable prevalence of ARGs and multiresistant microbiomes in the coyote population sampled were determined. The analysis of data through geo-reference systems has enabled the identification of associations between specific genes and geographic characteristics. This information facilitated the formulation of pivotal recommendations for the construction of antibiotic resistance mitigation strategies, particularly within the classes of antibiotics evaluated in the sampled areas.

Additionally, this study provides the first mitochondrial genetic data of the D-loop gene in Costa Rica for the species *C. latrans* and gray fox (*Urocyon cinereoargenteus*). This allows a preliminary analysis of the population connectivity of the coyotes sampled, providing baseline data for future studies on the population health of this species.

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-Por seguir viendo crecer sus sueños como vieron crecer el mío-

Mil gracias siempre.

Índice

Lista de cuadros

Descriptores

Resistencia bacteriana a los antibióticos:
Capacidad de las bacterias para resistir los efectos de los antibióticos, generalmente debido a mutaciones genéticas o la adquisición de genes de resistencia.

Genes de resistencia a los antibióticos (ARGs): Genes que proporcionan a las bacterias la capacidad de resistir los efectos de los antibióticos, permitiendo su supervivencia y proliferación.

One Health: Enfoque interdisciplinario que reconoce que la salud de las personas está conectada con la salud de los animales y el medio ambiente, promoviendo esfuerzos colaborativos para mejorar la salud global.

Bioindicadores: Organismos o comunidades de organismos que se utilizan para evaluar la salud de un ecosistema o para detectar contaminantes.

Especies centinela: Organismos que, debido a su alta sensibilidad y exposición a agentes patógenos, se utilizan para detectar la presencia de enfermedades emergentes y reemergentes en un ecosistema. Actúan como indicadores tempranos de cambios en la salud ambiental y pueden ayudar a predecir brotes de enfermedades que podrían afectar a otras especies, incluyendo a los humanos.

Antropogénica: Conjunto de acciones y procesos realizados por los seres humanos que afectan el medio ambiente.

Introducción general

La quimioterapia de agentes antimicrobianos inició con el descubrimiento de la inhibición del crecimiento de *Staphylococcus aureus*, dando origen al primer antibiótico, nombrado Penicilina (Saga & Yamaguchi, 2009; Yang et al., 2018). Descubrimiento que abrió paso a la implementación, por casi un siglo, de innovación en fármacos naturales y sintéticos. Llevando a tener aproximadamente 16 clases de antibióticos disponibles en la actualidad (Obando-Pacheco et al., 2020; Zhuang et al., 2021).

Los antibióticos tienen la función de inhibir el crecimiento bacteriano, interviniendo según la clase de antibiótico en cualquiera de los procesos celulares esenciales para la bacteria, como lo son la síntesis de la pared celular, la función de la ADN girasa, la ARN polimesara, la elongación del ARN mensajero (mARN), la síntesis proteínas (afectando las subunidades ribosomales o el ARN de transferencia (tARN), logrando detener el crecimiento y llevando a la bacteria a la muerte celular (Mohr, 2016). Es por esta razón que las bacterias han evolucionado buscando reducir o eliminar la eficacia de estos medicamentos. Adaptación originada por la incorporación de genes de resistencia a los antibióticos (ARGs) en su propio ADN, los cuales, brindan la ventaja de poder volverse resistentes a los agentes antimicrobianos, permitiendo expandir su nicho ecológico tanto en espacios nosocomiales como ambientales (Begum et al., 2021; Garza-Ramos et al., 2009).

La resistencia a los antibióticos es el resultado esperado de la interacción de los organismos con su entorno y debe ser evaluada desde dos perspectivas: primero, como mecanismo evolutivo de adaptación natural, como lo demuestra el hallazgo de ARGs a los β-lactámicos, tetraciclinas y vancomicina en sedimentos congelados de 30.000 años de antigüedad (D'Costa et al., 2011) y segundo, desde un enfoque clínico, donde las bacterias expuestas a medicamentos por debajo de la concentración bacteriana mínima sufren una presión de selección mayor, que a su vez aumenta el riesgo de que la bacteria desarrolle una resistencia al fármaco (Richardson, 2017).

La forma en que estos genes se transmiten entre las bacterias permite una clasificación de los mismos en intrínsecos y extrínsecos. La transmisión intrínseca indica una evolución vertical,

gracias a los procesos de fisión binaria o mutaciones espontáneas y los materiales genéticos extra cromosómicos procedentes de otras bacterias. Por otra parte, se considera la transmisión extrínseca o evolución horizontal la transferencia de elementos genéticos móviles (plásmidos, transposones e integrones) transmisibles a través de uno de los tres mecanismos genéticos: conjugación (implicando el contacto de célula a célula para el intercambio de ADN), transformación (la absorción de ADN desnudo del ambiente) o transducción (ADN transferido de una célula bacteriana a otra por virus) (Hu et al., 2016; Skarżyńska et al., 2020; Tenover & McGowan, 2008; Ugalde-Muñoz, 2007).

La adquisición de estos genes le brinda a la bacteria la capacidad de desarrollar diversos mecanismos bioquímicos capaces de resistir la acción inhibitoria de los antibióticos, ya sea mediante la presencia de enzimas hidrolíticas que inactivan el fármaco, la modificación del sitio de entrada de las proteínas de unión que emplea el medicamento, la alteración del sitio objetivo del antibiótico, cambios en la permeabilidad de la membrana bacteriana y la activación de bombas de eflujo activo hacia los principios activos (Begum et al., 2021; Rossolini et al., 2017).

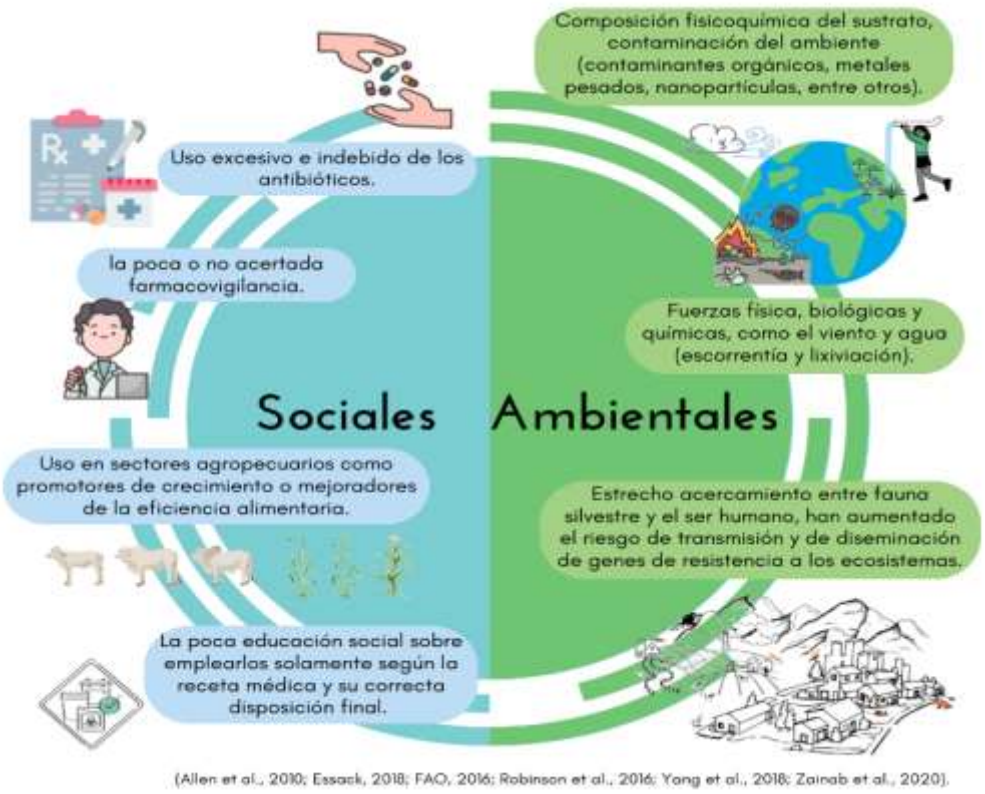
La resistencia de las bacterias a los antibióticos se ha perpetuado en el tiempo debido a la rápida evolución adaptativa que tienen y al bajo costo energético que tiene la permanencia de estos genes dentro de las bacterias. Cualidades que aumentan la posibilidad de que estas adquieran una colección de genes a través del tiempo, denominándose resistoma. Proceso que le brinda la capacidad de convertirse en una superbacteria y, por ende, aumentar el riesgo en la salud pública (Klein et al., 2018).

Es importante reconocer el limitado número de principios activos disponibles actualmente para el tratamiento de infecciones bacterianas. Dado que la mayoría de los fármacos disponibles en el mercado actualmente se descubrieron a mediados o finales del siglo XX. Compuestos que son ampliamente usados en humanos, animales y plantas, que al ser análogos entre sí potencializan la presión de transmisión entre actores (Begum et al., 2021; Essack, 2018). Adicionalmente son múltiples los factores que han elevado la selección de presión de las bacterias, desde acciones sociales, como el inadecuado uso de las recetas

médicas, el uso de antibióticos como promotores de crecimiento, continuando con condiciones ambientales como los procesos de escorrentía o contaminación ambiental, entre otros (Gráfica 1). Factores que contribuyen en conjunto a que la resistencia a los antibióticos se considere hoy en día una enfermedad emergente global (Wester et al., 2017).

Gráfica 1

Factores sociales y ambientales que han elevado la resistencia a los antibióticos.



Fuente: Elaboración propia, Adaptado de (Allen et al., 2010; Essack, 2018; FAO, 2016; Robinson et al., 2016; Yang et al., 2018; Zainab et al., 2020).

La presencia de resistencia bacteriana a los antibióticos en el medio ambiente desencadena una serie de consecuencias tales como un aumento de procesos clínicos severos y crónicos, incrementos de tasas de mortalidad, pérdidas de producción, reducción en la seguridad alimentaria, aumento en costos de tratamiento y atención médica entre otros (de León-Rosales et al., 2015; FAO, 2016; Prestinaci et al., 2015). El Banco Mundial en el 2016 indicó que "un escenario de alta resistencia (…) podría causar a los países de bajo ingreso una

pérdida de más del 5% del producto interno bruto (PIB) y empujar a 28 millones de personas, la mayoría de los países en desarrollo, a la pobreza para 2050".

Esta mirada de preocupación llevó a que en ese mismo año, la Asamblea General de las Naciones Unidas (AGNU), conformada por la Organización Mundial de la Salud (WHO, por sus siglas en inglés), en colaboración con la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) y la Organización Mundial de Sanidad Animal (OIE), declararan una política de creación de un Plan de acción mundial para luchar contra la resistencia a los antimicrobianos (Organización Mundial de la Salud, 2016), esfuerzos a los cuales se unió en el 2019 la ONU con su programa para el medio ambiente (UNEP, por sus siglas en inglés) (WHO et al., 2022). Esta directriz promueve un enfoque interdisciplinario para abordar la problemática, proporciona información de monitoreo global y en tiempo real, lo que facilita la orientación en acciones de mitigación que contribuyan a la salud humana y a la preservación de los ecosistemas reconociendo que el medio ambiente es un componente clave en la tríada de One Health y que su vigilancia es crucial para predecir la aparición de patógenos resistentes emergentes (Founou et al., 2017; Klein et al., 2018; Medina-Pizzali et al., 2021; Wester et al., 2017; Zhuang et al., 2021).

Al mismo tiempo, frente a la necesidad de realizar un monitoreo asertivo de esta emergencia se han implementado diferentes métodos de detección de resistencia bacteriana. Inicialmente, se utilizaron técnicas fenotípicas mediante análisis de susceptibilidad por métodos de difusión o dilución, los cuales permiten identificar la expresión de resistencia y el agente portador, facilitando la comparación entre especies. Sin embargo, estas técnicas presentan limitaciones debido al tiempo requerido y a la necesidad de cultivar la bacteria de interés (Anjum et al., 2021; Organización Panamericana de la Salud, 2023). En los últimos años, los métodos moleculares han ganado relevancia, ya que amplían el alcance de la evaluación y permiten una caracterización detallada de los genes de resistencia, sus factores de virulencia, su ubicación genética y la recopilación de datos para un monitoreo global. Facilitando la compresión en la distribución y evolución de la resistencia a los antibióticos en diversas poblaciones bacterianas, contribuyendo significativamente a estudios epidemiológicos mediante bibliotecas de registro de información bioinformática como The Comprehensive

Antibiotic Resistance Database (CARD) y The National Center for Biotechnology Information (NCBI) (Anjum et al., 2021; Garza-Ramos et al., 2009; McDermott & Davis, 2021; *National Center for Biotechnology Information*, s. f.; Xu et al., 2019).

En el componente ambiental las investigaciones reportan alta presencia de genes de resistencia a los antibióticos en múltiples especies de vida silvestre en diferentes ecosistemas y continentes (Bamunusinghage et al., 2022; Ramey, 2021; Swift et al., 2019; Vittecoq et al., 2016). Presencia asociada a la presión antropogénica, dado que los antibióticos no se metabolizan por completo después de su administración, llevando a que sus metabolitos se excretan sin cambios a través de la orina y las heces (Truong et al., 2021), impregnándose en diversos entornos y que, debido a la sobreexplotación de recursos naturales, cambio de uso de suelo, entre otros, se incrementa las posibilidades de contacto entre los ARGs y la fauna silvestre (Aguilar-Vargas et al., 2022; Czatzkowska et al., 2022; Robinson et al., 2016; Skandalis et al., 2021).

Por lo tanto, la adquisición de estos genes en la fauna silvestre puede ocurrir a través de diversas rutas de contacto, como el agua contaminada, fuentes de alimentos, proximidad a animales domésticos, o mediante la diseminación a través de la cadena trófica (Lerner & Berg, 2017; O'mahony et al., 2006; Worsley-Tonks et al., 2020). Lo que permite que la fauna silvestre actúe como centinela de la salud ecosistémica, proporcionando información sobre la ocurrencia espacio-temporal de múltiples contaminantes y una detección temprana de alteraciones en el ambiente, como la reemergencia o emergencia de ciertos patógenos (Aguilar-Vargas et al., 2022; Allen et al., 2010; Angulo et al., 2018; Baldi et al., 2019; Ballash et al., 2021; Blanco-Peña et al., 2017; Brealey et al., 2021; Esquivel Aguilar, 2004; Gamboa et al., 2004; Kozak et al., 2009; Oviedo-Bolaños et al., 2021; Ramey, 2021; Rodríguez-Rodríguez et al., 2007; Rojas Jiménez, 2018; Rojas-Jiménez et al., 2019; Ugalde-Muñoz, 2007).

En Costa Rica, hasta la fecha se han realizado estudios que abordan la problemática de resistencia a los antibióticos desde un enfoque integral de One Health, analizando la detección en fauna de presencia o ausencia de esta resistencia por medio técnicas

microbiológicas o de biología molecular abarcado diversas especies, como *Alouatta palliata, Ateles geoffrogyi, Saimiri Oerstedii, Procyon lotor, Panthera onca, Puma concolor, Columba livia, Oreochromis niloticus y Tapirus bairdii*, en una variedad de entornos que incluyen cautiverio, hábitat urbano, sitios de producción acuícola y zonas de reserva (Angulo et al., 2023; Baldi et al., 2019; Blanco-Peña et al., 2024; Blanco-Peña et al., 2017; Gamboa et al., 2004; Oviedo-Bolaños et al., 2021; Rodríguez-Rodríguez et al., 2007; Rojas Jiménez, 2018; Rojas-Jiménez et al., 2019, 2022).

Esta investigación realizó un panel de hasta nueve ARGs diferentes que codifican la resistencia a cinco clases diferentes de antimicrobianos con diferentes mecanismos de acción de resistencia a la acción farmacológica (Tabla 1). Seleccionados a su vez por ser ampliamente empleados en medicina humana, agricultura y medicina veterinaria: tetraciclinas (*tet*W, *tet*Q y, *tet*Y), sulfonamidas (*sul*I y *sul*II), fenicoles (*cat*AI y *cat*AII), quinolonas (*qnr*S) y betalactámicos (bla_{TEM}).

Tabla 1

Mecanismos de resistencia a la acción farmacológica presentes en los nueve ARGs evaluados este estudio (tetW, tetQ, y tetY, sulI, sulII, catAI, catAII, qnrS y bla_{TEM}), pertenecientes a cinco clases de antibióticos (Tetraciclinas, Sulfonamidas, Cloranfenicoles, Quinolonas y Betalactámicos).

Clase de antibiótico	Mecanismo de acción del antibiótico	Gen de resistencia	Mecanismo de acción del gen de resistencia
Tetraciclinas	INHIBICIÓN DE LA FORMACIÓN PROTEICA. Bacteriostáticos, inhibiendo la síntesis de proteína, en el ribosoma 30S, previniendo la unión del aminoácido t-RNA y la elongación y formación de proteínas. Ingresa por difusión pasiva a través de porinas	*tet*W, *tet*Q, y *tet*Y	MECANISMOS DE EFFLUX Y PROTECCIÓN DE LA DIANA EN EL RIBOSOMA, con unión reversible de trifosferasa de guanosina. *tet*Y**:** *efflux* detectado en Gram-, Enterobacterias, asociado con el ADN plasmídico. *tet*W**:** alteración ribosomal en Gram+ *Streptococcus* y *Staphilococcus* y Gram– Enterobacterias, asociados a

			procesos de conjugación de ADN. *tet*Q**:** Protección ribosomal en Gram+ *Streptococcus* y Bacteroides, ligado a cromosoma, plásmidos y transposones, de procesos de conjugación.
Sulfonamidas	INHIBICIÓN DE VÍA METABOLICA. Inhiben competitivamente la Dihidropteroato sintasa (DHPS), enzima que cataliza la formación de dihidropteroato a partir de ácido p-amino benzoico y 6-hidroximetil- 7,8-dihidropterin pirofosfato, un paso temprano de la vía de síntesis de folato.	*sul*I, *sulI*I	ALTERACIÓN DEL SITIO OBJETIVO DEL ANTIBIÓTICO, por mayor producción de ácido p-amino benzoico y la producción de DHPS resistentes o con afinidad reducida a las sulfonamidas. Mecanismo ligado a los integrones tipo 1 de las bacterias Gram–.
Cloranfenicoles	INHIBICIÓN DE LA FORMACIÓN PROTEICA. Afecta al ribosoma 50S inhibiendo la síntesis proteica e inhibiendo la peptidotransferasa.	*catA*I, *catA*II	INACTIVACIÓN DEL FÁRMACO, por la producción de la enzima cloranfenicol acetiltransferasa. Enmascaran el sitio de unión en el ribosoma produciendo rRNA metilazas.
Quinolonas	INHIBICIÓN DE VÍA METABOLICA. inhibición enzimática de ADN girasa o la topoisomerasa IV. Enzimas esenciales en la replicación, segregación, transcripción, recombinación y reparación cromosómica.	*qnr*S	MECANISMO PROTECCIÓN DE LA DIANA EN EL RIBOSOMA. Tiene varios mecanismos: I. Mutación del sitio diana. II. Permeabilidad reducida o eflujo activo. III. Protección mediante proteínas específicas. IV. Inactivación de fármacos.

			qnr: Producción de proteínas que protegen a la topoisomerasa de la interacción con las quinolonas, mecanismo transferible codificado por plásmidos, principalmente reportado en cepas de Enterobacterias.
Betalactámicos	INHIBICIÓN DE LA SINTESIS DE LA PARED CELULAR. Inhibe la cadena de péptidoglicanos	*bla*$_{TEM}$ *(Molécula Clase A, caracterizada por codificarse por plásmido siendo de amplio espectro, muy frecuente en entornos clínicos).*	INACTIVACIÓN DEL FÁRMACO. I. Producción de B-lactamasa, destruyendo el anillo B-lactamico. II. Presencia de proteínas de unión de penicilina para dar baja afinidad a los antibióticos (Principal vía de las bacterias nosocomiales, asociado a *erm*). III. Bombas de efusión o disminuyendo la permeabilidad de la membrana. Se clasifican según 4 moléculas (A, B, C y D). Las cuales solo B confiere zinc para su actividad y las otras tres serinas.

Nota: Tabla de elaboración propia, Adaptado de: (Alcock et al., 2023; Allen et al., 2010; Rossolini et al., 2017; Tenover, 2006; Thomas et al., 1997; Vicente & Pérez-Trallero, 2010).

El monitoreo se llevó a cabo en dos áreas de conservación de Costa Rica, Área de Conservación Central (ACC) y Área de Conservación Guanacaste (ACG) bajo el amparo del permiso R-CM-UNA-004-2022-OT-CONAGEBIO. El ACC abarca una superficie de 650.918 hectáreas, comprende la Estación Biológica La Selva, la Reserva Biológica Alberto Manuel Brenes, el Parque Nacional Braulio Carrillo, el Parque Nacional Quetzales y la Reserva Forestal Central Cordillera Volcánica. La región se distingue por su topografía elevada, densa cobertura boscosa y una red de ríos, además de la ganadería extensiva (Angulo et al., 2023; Chassot et al., 2009; SINAC, 2019). La segunda zona fue el ACG con 163.000

hectáreas, situada al noroeste de Costa Rica, encontrando el Parque Nacional Santa Rosa, el Parque Nacional Guanacaste, el Parque Nacional Rincón de la Vieja, el Refugio Nacional de Vida Silvestre Junquillal y la Estación Forestal Experimental Horizontes. Esta región abarca una gama de ecosistemas representativos de climas tropicales, incluyendo arrecifes de coral, bosque seco, bosque tropical, bosque nuboso y bosque lluvioso (Janzen & Hallwachs, 2020; SINAC, 2019). Estas dos áreas han sido sometidas a actividades humanas, incluyendo la utilización intensiva de agroquímicos y fertilizantes, el desvío de los cursos de los ríos y la contaminación de las fuentes de agua (Costa Rica. Ministerio de Ambiente y Energía, 2001).

Finalmente, la especie empleada como centinela para este estudio correspondió al coyote (*Canis latrans*), meso-carnívoro, presente en zonas limítrofes, con grandes desplazamientos de forma solitaria y gran plasticidad a múltiples ambientes con cambios antropogénicos y preferencia de áreas abiertas (Carazo-Salazar et al., 2020). Atributos que le han permitido colonizar nuevas regiones, llevando a tener una amplia distribución geográfica desde Canadá hasta Panamá (Monroy-Vilchis et al., 2020) movimiento que a su vez modifica la estructura y función de los ecosistemas invadidos, causando competencia, depredación y propagación de enfermedades y parásitos (Stohlgren & Schnase, 2006).

Para el caso de Costa Rica, se tienen reportes históricos de la presencia de coyotes en la región de Guanacaste desde el Pleistoceno, inclusive hay registros fósiles de los mismos en la zona (Hody & Kays, 2018). Pero desde los años 40 y 60 se ha observado una ampliación paulatina y considerable en la distribución de la especie, teniendo reportes actuales en el Valle Central y zonas más hacia el sur del país, observando la ubicación de la especie en las filas de montañas, zonas fronterizas entre bosque, charrales, potreros y cultivos (Carazo-Salazar et al., 2020; Hurtado et al., 2018; Lloyd-Alcock, 2020; Picado-Umaña et al., 2009).

Los estudios han indicado que este comportamiento de expansión puede estar influenciado por dos situaciones: características propias del coyote y cambio de uso del suelo. La primera, debido a su alto potencial reproductivo, hábitos alimenticios versátiles y a sus amplios requerimientos territoriales, ya que se ha reportado desplazamientos diarios en promedio de 2 a 8 km, con un máximo de 35 km y con rangos de hogar que cubren los 72 Km^2 (Lloyd-

Alcock, 2020; Vaughan, 1983) y la segunda, por la tala de los bosques del Pacífico centroamericano, para el crecimiento de monocultivos o ganadería que con llevaron a la formación de corredores de paso que afectaron a grandes depredadores, promoviendo a su vez, la colonización del coyote a nuevos espacios, brindando una gran cercanía de estos a las áreas urbanas (Literák et al., 2012). Esto último favoreció la inclusión de animales de corral en su espectro de alimentación, confirmado por reportes en foto trampeo en la región noreste y norcentral de Costa Rica. En el 2024 se reportó presencia de la especie en el Parque Nacional Chirripó, indicando que el coyote está presente actualmente en todas las latitudes del país. (Azofeifa-Romero et al., 2024; Cove, 2012; Hody & Kays, 2018; Hurtado et al., 2018; Picado-Umaña et al., 2009).

Para el desarrollo de la investigación se requirió realizar la confirmación de la especie colectada. Proceso realizado mediante la amplificación por reacción en cadena de la polimerasa (PCR) en punto final y posterior secuenciación de Sanger en dos direcciones de la región de control (D-Loop) del ADN mitocondrial (ADNmt) con los primers (SIDL 5-TCTATTTAAACTATTCCCTGG-3, H3R 5-CCTGAAGTAGGAACCAGATG-3) de (315 a 401 pb) (De Barba et al., 2014). Se obtuvo que un 67% del material colectado correspondía a coyote (*C. latrans*), mientras que el porcentaje restante indicó muestreo de otros canidos como zorro gris (*Urocyon cinereoargenteus*) y perro doméstico (*Canis lupus familiaris*). Las secuencias genéticas obtenidas fueron depositadas en Centro Nacional para la Información Biotecnológica (en inglés: National Center for Biotechnology Information [NCBI]), incorporando la primera información de coyote y zorro gris de Costa Rica, y complementando los registros internacionales de linajes genéticos para las dos especies de canidos silvestres detectados en este estudio.

Posteriormente, para el monitoreo de resistencia bacteriana a los antibióticos, en primera instancia se determinó la presencia de material genético bacteriano en las muestras de coyotes, a través de una PCR dirigida a la región (16S rRNA) (27F 5-AGAGTTTGATCCTGGCTCAG-3, 1492R 5-GGTTACCTTGTTACGACTT-3) (C. Miller et al., 2013; Srivastava et al., 2008). Finalmente, a las muestras positivas con el tamaño esperado (250 a 300 pb) se les cuantificó y detectó la presencia los ARGs (*tet*W, *tet*Q, *tet*Y, *sul*I, *sul*II, *cat*AI, *cat*AII, *qnr*S y bla_{TEM}) a través de PCR en tiempo final y mediante qPCR,

buscando analizar esta información mediante herramienta de análisis espacial correlacionando la presencia de ARGs con variables antropogénicas y ambientales.

La implementación de la metodología descrita anteriormente permitió obtener resultados significativos, a partir de los cuales se redactaron tres artículos científicos con los objetivos claramente diferenciados propuestos en esta investigación. El primer artículo se centró en la caracterización de la presencia o ausencia de siete ARGs en muestras de heces de coyotes recolectadas en las Áreas de Conservación de Guanacaste y Central, explorando la clasificación taxonómica de los ARGs amplificados y proporcionando información de relevancia internacional para monitoreos epidemiológicos a gran escala. El segundo artículo se realizó en la modalidad de "short communication", y se centró en la presencia de ARGs del zorro gris (*Urocyon cinereoargenteus*) colectado en la zona norte del ACG. Por último, el tercer artículo se enfocó en la cuantificación de nueve ARGs mediante la técnica de PCR en tiempo real (qPCR), complementando el diagnóstico con un análisis espacial de diversos factores antropogénicos y ambientales que interactúan con las muestras recolectadas a lo largo del año 2022 en las Áreas de Conservación de Costa Rica mencionadas.

Conclusiones generales

1. Este estudio confirma por primera vez en CR presencia de ARGs *(tetW, tetQ, y tetY, sulI, sulII, catAI, catAII, qnrS y bla$_{TEM}$)* en coyotes en Costa Rica con un alto porcentaje de microbiomas multirresistentes.

2. Los análisis de PCR punto final mostraron que el 97% de los animales muestreados (n=35) dieron positivo para al menos uno de los siete ARG evaluados. Geográficamente, se observó mayor prevalencia de ARGs en ACC (78%) frente al (54%) en ACG. Los genes *bla*$_{\text{TEM}}$ (97%), *sul*I (95%) y *tet*W (94%) fueron los más comunes en ambas áreas.

3. Los análisis de PCR en tiempo real detectaron al menos uno de los nueve genes en todas las muestras evaluadas (n=35). Cuatro muestras (11%) mostraron amplificación para todos los genes, y el 91% presentó un microbioma multirresistente. Todos los análisis resultaron positivos para los genes *tet*W, *tet*Q y *sul*I. La muestra 54 de ACG presentó la mayor concentración de ARG en tres de los genes evaluados (*tet*Q, *catA*I y *sul*I).

4. Según los datos de cuantificación relativa de genes basado en el número de bacterias de cada muestra, se observó que hay diferencia en la cantidad de genes registrados por área de conservación y entre los mismos genes ($F= 3.944$; $p= 0.0084$). *sulI*, *tet*Q y *tet*Y presentan diferencia con relación a *catA*II, siendo esta última la que presentó menor abundancia. Mientras que, los genes *sul*II y *tet*Y tienen diferencias en su concentración entre ambas áreas de conservación (Dunnpost huc, $p< 0.05$), lo que puede evidenciar distintos tipos de contacto con los antibióticos.

5. En análisis espacial permitió explorar la relación entre la cuantificación de genes por muestra con las posibles fuentes de contacto, indicando que los genes *catA*I, *catA*II, *tet*Y y *tet*W están asociados a las carreteras, *sul*II a ríos y *bla$_{TEM}$* a sistemas productivos de vacas y cerdos.

6. El coyote se confirma como una especie centinela eficaz para monitorear la interfaz entre ambientes humanos y naturales, como bioindicador ambiental de contaminación antrópica, desempeñando un papel crucial en la vigilancia de la salud ecosistémica y en la evaluación de la eficacia de planes de acción y monitoreo de la resistencia a los antibióticos en Costa Rica.

7. Se registró en el Centro Nacional para la Información Biotecnológica (en inglés: National Center for Biotechnology Information [NCBI]), las secuencias de los ARGs

analizados: PP693909-PP693925, PP757467-PP757487. Información clave para robustecer análisis epidemiológicos sobre la dinámica de los genes de resistencia a los antibióticos, especialmente en Costa Rica y Centroamérica.

8. Por primera vez, se utilizaron secuencias D-loop de ADN mitocondrial para establecer linajes maternos de poblaciones de coyotes y zorros grises en Costa Rica. Estas secuencias, también registradas en el NCBI, obtuvieron los números de registro para el gen D-loop de *C. latrans* (PP454122-PP454156) y *U. cinereoargenteus* (PP974326, PP974327 y PP974329). Esta información bioinformática servirá como base para futuros estudios de genética poblacional en estas especies en el país.

9. En las secuencias mitocondriales de coyotes se exploró preliminarmente la conectividad genética entre las muestras colectadas. El análisis filogenético, basado en el posicionamiento taxonómico, mostró la formación de dos clústeres. El primero incluyó la mayoría de las muestras del volcán Irazú (ACC) y una muestra de Horizontes (ACG), con un soporte de rama del 98, y agrupadas junto a *C. latrans* de Texas, Luisiana, Ohio y Kentucky. En contraste, el segundo clúster consistió principalmente en secuencias de ACG y una de Irazú (ACC), cercanas a especímenes registrados en Carolina del Norte, los Grandes Lagos, Wisconsin y Canadá.

10. La variación genética evaluada en 33 muestras analizadas por el programa DNASP mostró que los dos hábitats muestreados (AGG y ACC) presentan tres haplotipos, con una diversidad de haplotipos de Hd: 0,606, una varianza de 0,0024 y una diversidad nucleotídica de π 0,0099. Resultados que indican una baja, pero presente diferenciación genética entre las dos áreas colectadas.

11. En cuanto a divulgación científica del proyecto contó con la participación en los congresos internacionales: VI Encuentro Bienal Centroamericano y del Caribe UNA, Segundo Encuentro Latinvets “Claudia Brieva” y el II Congreso Regional en Ciencias de la Tierra y el Mar y V Congreso Internacional de Geografía Urbana, actividades en donde se resaltó la importancia de un enfoque integral para afrontar la crisis de resistencia a los antibióticos. Presentando una metodología de diagnóstico oportuno y de fácil replicabilidad para la detección de genes de resistencia antimicrobiana (ARGs) en bacterias ambientales.

Recomendaciones generales

- Promover talleres desde la extensión universitaria interdisciplinaria, en donde se promueva el uso de prácticas culturales y técnicas adecuadas en los sistemas de producción pecuaria mejorando el bienestar animal, reduciendo el riesgo de enfermedades, brindando a su vez técnicas de mejora en higiene, tratamientos preventivos y estimular el uso de antibióticos luego de tener diagnóstico clínico por un profesional. Enfocando esfuerzos de control y buen uso en los antibióticos pertenecientes a las familias de las tetraciclinas, sulfonamidas y quinolonas.
- Complementar y estimular capacitaciones por parte del Ministerio de Agricultura y Ganadería y del Ministerio de Salud Pública de Costa Rica, con actividades *in situ*, desarrollando estrategias de educación sobre la importancia de los antibióticos, como emplearlos adecuadamente, el tiempo de retiro adecuado, como realizar un correcto almacenamiento y eliminación de los mismos.
- Buscar mejorar el mecanismo de recolección de envases vacíos, indicando puntos de acoplamiento y disposición regular de estos fármacos, fortaleciendo a la construcción de una política pública de manejo adecuado de residuos hospitalarios.
- Integración de los datos obtenidos en investigaciones ambientales en el sistema de monitoreo a nivel nacional, con el fin de direccionar las estrategias de mitigación desde un enfoque One Health.
- Promover la expansión del monitoreo de genes de resistencia a los antibióticos en diversas áreas de conservación mediante las técnicas moleculares adelantadas en esta investigación.
- Fomentar la investigación continua sobre la diversidad genética y estructura poblacional de coyotes y zorros grises en Costa Rica por medio de análisis de detección de número de alelos por locus y red de haplotipos.
- Los hallazgos de variación genética deberían ser corroborados a través de una muestra más grande, dado que si persiste esta baja diversidad disminuye su capacidad de adaptarse ante potenciales cambios ambientales, considerando el posible impacto en la salud de la población y el riesgo de estar en contacto con contaminantes como los ARGs.

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Artículo I: Detection of antimicrobial resistance genes (ARGs) in coyotes (*Canis latrans*) from two conservation areas of Costa Rica: An approach from conservation medicine

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Environmental Research

Detection of antimicrobial resistance genes (ARGs) in coyotes (*Canis latrans*) from two conservation areas of Costa Rica: An approach from conservation medicine

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ABSTRACT

Researchers documented evidence of microbes harboring antimicrobial resistance genes (ARGs) living on and in various wildlife species and across environmental niches. Consequently, each country must tailor AMR surveillance, control programs, and policies to meet their needs. In this study, we examined the presence of these genes within the framework of public health and conservation, focusing on a wildlife sentinel species, from a conservation medicine perspective, which integrates actions from all stakeholders involved in AMR. Our study monitored two conservation areas in Costa Rica by collecting 35 fecal samples from coyotes (*Canis latrans*), a meso-carnivore adaptable to diverse environments. We collected samples opportunistically from the Guanacaste (ACG) and Central (ACC) conservation areas between March and August of 2022. We confirmed fecal samples matched coyote origins through the analysis of the extracted mitochondrial DNA (mtDNA) displacement loop (D-lopp) region. We monitored the microbial DNA extracted from the coyote fecal samples for a total of seven ARGs. 97% of animals sampled were positive for at least one ARG. *bla*$_{\text{TEM}}$ (97%), and *sul*I (95%) were the most prevalent. Regarding geographic distribution, ARGs were more prevalent in areas with higher human population density (79% in ACC compared to 54% in ACG). What's more is the coyote DNA extracted from the fecal samples were used to conduct the country's first preliminary genetic diversity analysis, revealing a low number of haplotypes (n = 3), haplotype diversity (Hd = 0.606),

and nucleotide diversity (π = 0.0099). This study significantly providing valuable information for controlling AMR in Costa Rica.

KEYWORDS: Central America, DNA barcoding, environmental pollution, taxonomic placement, wildlife.

HIGHLIGHTS

- ARG pollution significantly affects Costa Rica's conservation areas.
- Collected coyote feces show multidrug-resistant microbiomes.
- First report of ARGs in coyotes in Central America.
- Preliminary genetic diversity data for *Canis latrans* in Central America.

1. INTRODUCTION:

The coyote (*Canis latrans*) is a widely distributed species present in border zones, buffer areas between conserved ecosystems, and human settlements, with a habitat range of 72 km^2 for Costa Rica. It exhibits remarkable plasticity to many environments including those with anthropogenic changes [1,2]. This meso-carnivore has expanded into new territories, often close to urban areas, favoring the inclusion of livestock animals in its feeding spectrum due to the deforestation of the Central American forests and the growth of monocultures and livestock in the country [3,4]. These characteristics position the coyote as a prime candidate to serve as the main terrestrial sentinel of emerging diseases in the country's conserved ecosystems [5].

Habitat fragmentation due to urban expansion, degradation of the natural environment, expansion of agricultural production, and loss of high depredators have increased the presence of coyote populations in buffer zones [6]. Factors that cause alterations in population dynamics could represent a decrease in genetic diversity, which reduces the ability to fight diseases and deal with other threats [7,8]. What's more, the animals' natural microbiota is affected by exposure to antibiotic resistance genes (ARGs), thus altering the balance of beneficial microorganisms and harming the animals' immune system. For example, altered microbiota and decreased genetic diversity can increase susceptibility to

infections, changing the flow of nutrients within the food chain and causing immunosuppression [9,10].

Evaluating AMR in the environment is crucial due to its role as a disseminator and point of evolution of ARGs [11]. The environment exhibits irregular dispersals of these genes, which can be highly clinically relevant and possess a significant capacity to migrate across environmental gradients influenced by human activities [12,13]. Furthermore, understanding the genetic diversity of species and their connective population contributes to the generation of phylogenetic information that facilitates future studies of biodiversity and species population structure. From a conservation medicine perspective, knowledge of genetic diversity can influence a species' ability to resist diseases (such as emerging ARGs), adapt to environmental changes, and survive climate change events [14].

For these reasons, the selective pressure caused by human presence and the interaction between coyotes and pets, livestock, and urban areas can promote changes in the population structure and gene exchange among bacteria. This gene exchange may also contribute to the spread of antibiotic resistance, serving as reservoirs or disseminators and potentially contributing to the evolution of "super bacteria" [15–17]. Numerous global studies have documented the widespread spectrum of ARGs in wildlife, emphasizing the critical need for continuous monitoring of antibiotic resistance in diverse ecosystems [18–21]. Costa Rica has emerged as a leader in Central America in investigating its AMR context [22], with several studies reporting the presence of ARGs in wildlife and various environments [18,23–31].

This study analyzed two conservation areas of Costa Rica: Guanacaste and the Central Conservation Area through *C. latrans* feces collected in 2022. The Endpoint PCR molecular technique was employed to conduct a preliminary analysis in the country of population genetic diversity and monitor the presence of ARGs from four different families: sulfonamides (*sul*I), phenicols (*cat*AI, *cat*AII), beta-lactams (bla_{TEM}) and tetracyclines (*tet*Q, *tet*W, and *tet*Y). Sanger sequencing technology was used to analyze amplicons. For the first time in Central America, our study used ARG analyses and genetic data on generalist mammal coyotes to report habitat alteration due to anthropogenic activities. This research aims to generate valuable information that will guide improvements to the conservation plans and ecosystem protection measures carried out in Costa Rica from a conservation medicine perspective. In the future, monitoring genetic diversity based on molecular structure and the presence of ARGs over time will be crucial for evaluating the effectiveness of conservation strategies, mitigating the use of antibiotics, or adapting their use as needed. This perspective

can lead to a series of interconnected effects that impact human, animal, and ecosystem health.

2. METHODS

*2.1. **Study area***

The sampling was conducted in two conservation areas managed by the government of Costa Rica through the National System of Conservation Areas (SINAC). In the Guanacaste Conservation Area (ACG), the sample sectors included Santa Elena, Santa Rosa, Pocosol, and Horizontes. Samples were simultaneously collected in the Costa Rican high-altitude forests: Turrialba Volcano, Irazú Volcano, and the Cascajal de Vázquez de Coronado in the Central Conservation Area (ACC). Both regions are characterized by great biological diversity, including multiple ecosystems such as tropical forest, cloud forest, and rainforest, with the ACG also containing dry forests. Human agricultural activity surrounds these areas. The contrast between both regions is highlighted by the fact that the pressure due to population density is more significant in the ACC, as it is the closest and most accessible conservation area from the Central Valley, where the majority of Costa Rica's population resides [32] (Figure 1).

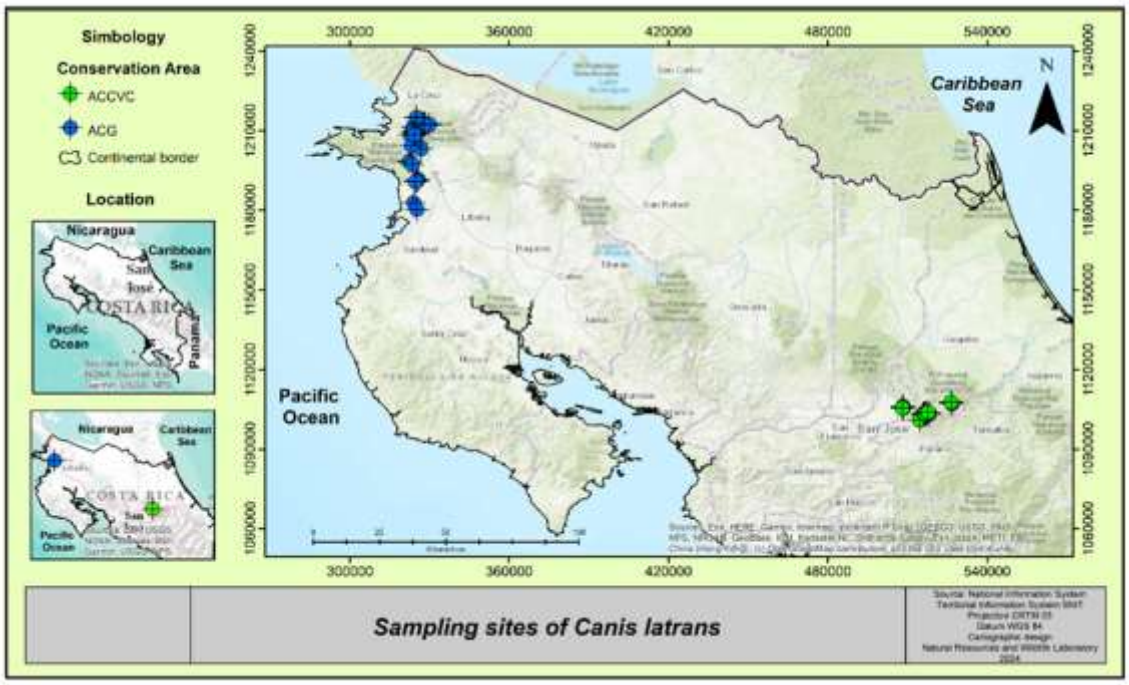

Figure 1. Sampling sites in the two conservation areas of Costa Rica, selected for this study: **A**. Guanacaste (ACG) North Pacific location. **B**. Central Conservation Area (ACC).

2.2. ***Sample collection***

Fecal samples were collected opportunistically between March and August 2022, according to the entry and collection permits obtained for the conservation areas (R-CM-UNA-004-2022 -OT-CONAGEBIO). The selection criteria for the identification and collection of biological material included the habitat and activity patterns of the species previously reported [2], physical characteristics, presence of nearby footprints, confirmation of the existence of the species using camera traps and criteria of the researcher (thermal, olfactory, and consistency sensation). Each fecal sample was described according to the morphological description of its components, including fruit, hair, bones, and seeds, and individually stored in 50 mL conical tubes appropriately labeled with identification (ID), date, and GPS coordinates. The samples were then transported, while maintaining the cold chain at 4°C, to the Regional Institute for Studies in Toxic Substances (IRET) for storage at -80°C until their subsequent analysis at the Genomic Analysis Laboratory (LAGen), School of Biological Sciences, Universidad Nacional, Costa Rica.

2.3. ***Fecal DNA* extraction**

DNA extraction was conducted using the QIAamp™ DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions. The concentration of extracted DNA was determined using a UV-visible microvolume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). The purity of the nucleic acids was assessed by measuring the absorbance coefficients A260/A280 and A260/A230. DNA integrity was evaluated through electrophoresis in 1% w/v agarose gel, using GelRed (Biotium) and GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific Inc.).

2.4. ***PCR amplification for species identification***

To confirm the species identity of the collected feces as *C. latrans*, short segments of the mtDNA control region were amplified by endpoint PCR using forward primer SIDL 5-TCTATTTAAACTATTCCCTGG-3 and reverse primer H3R 5-CCTGAAGTAGGAACCAGATG-3 [33]. PCR amplicon sizes of 315 to 401 base pairs (bp) were considered potentially positive for carnivorous species. These positive samples were purified by absolute ethanol precipitation to subsequently corroborate their molecular identity through Sanger sequencing using a 3500 genetic analyzer (Applied Biosystems®), BigDye™ Terminator V3.1 chemistry, and BigDye XTerminator™ purification kit, following the manufacturer's instructions. The sequences obtained were edited in the Geneious Prime® program (v.2023.2.1, Biomatters Ltd), and then analyzed with the online program BLASTn [34] available at the National Center for Biotechnology Information

(NCBI). The program was used to compare the sequences obtained with the GenBank database (http://www.ncbi.nlm.nih.gov).

2.5. ***Antibiotic resistance genes (ARGs) detection***

The presence of bacterial DNA was confirmed by amplifying the 16S rDNA gene using the primers 27F and 1492R in all positive fecal samples of *C. latrans* [35,36]. The amplification was considered positive for bacteria if the amplicon size was 250 bp to 300 bp. Then, bacterial DNA was analyzed by qPCR for the presence of the following ARGs; sulfonamides (*sul*I) [37], phenicols (*cat*AI, *cat*AII) [20,38], beta-lactams (bla_{TEM}) [39], and tetracyclines (*tet*Q, *tet*W, and *tet*Y) [20,40] (Table S1, Supplementary material). The Comprehensive Antibiotic Resistance Database (CARD), was used to identify ARG sequences to serve as positive controls for each primer set [41]. MacVector software v18.6 was used to analyze CARD control sequences and generate and test potential primer pairs and known primer pairs and their optimal PCR conditions. ARG Controls were synthesized by Integrated DNA Technologies as gblocks v0.91b program, double stranded DNA fragments, shipped dry and subsequently resuspended in nuclease-free water. These DNA control fragments were designed to amplify an internal portion of each selected ARG, for safety reasons, so as not to perpetuate amplification of whole ARGs and risk dissemination. Finally, representative PCR products from each ARG amplified from DNA extracted from fecal samples were selected and purified to corroborate their identity using Sanger sequencing technology.

2.6. ***Taxonomic placement***

A phylogenetic analysis was carried out by sequence alignment between the Genbank database and molecular data of this study. Initially, sequence matrices were created for each gene separately (D-loop and ARGs) by aligning them with MAFFT v7.490 [42]. Conserved blocks were then selected using less strict parameters in the gblocks, eliminating divergent or uninformative sites from each alignment [43]. To construct the taxonomic trees, IQ-TREE (https://www.hiv.lanl.gov/content/sequence/IQTREE/iqtree.html) multicore program was used [44]. Phylogenetic topology was constructed using the maximum likelihood (ML) algorithm, selecting the best substitution model using the Akaike information criterion (AIC). The model-selection method determined the best model for D-loop: K3Pu+F+I (AIC= 1137.521) and the ARGs: TVMe+G4 (AIC= 13631.302). The parameters used for phylogenetic inference of both trees were free rate heterogeneity and node support calculation derived from the ultrafast bootstrap method of 10,000 replicates. FigTree v1.4.4 program was used to visualize and edit the topology inference.

2.7. ***Genetic diversity analysis***

The genetic variation was evaluated in 33 individuals of the 35 *C. latrans* fecal samples through the determination of the number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π) [45,46]. This information provided a first preliminary approximation of genetic diversity information for coyotes in Costa Rica. A dataset was obtained with the Geneious Prime® software by MAFFT v7.490 alignment, using the iterative refinement method (G-INS-i) and the 1PAM parameter k=2 [42,47]. Subsequently, DnaSP, DNA sequence polymorphism software (v.2018.6.12) was used to show DNA sequence variation within the *C. latrans* population of this study [48].

3. RESULTS

3.1. ***Fecal morphology***

A total of 53 fecal samples were collected. They exhibited various components upon morphological description, including fruit, hair, bones, and seeds. Samples from ACG showed a significant proportion of nance fruit (*Byrsonima crassifolia*), giving them a yellowish-brown hue. In contrast, samples from ACC displayed a higher presence of hairs and bones, characterized by a predominant very dark brown color (supplementary material, Figure 1S).

3.2. ***Molecular identification of C. latrans in fecal DNA***

Of the 53 samples analyzed, 35 belonged to *C. latrans* (67%, 35/53), with an average of 360 bp, followed by 9 of domestic dog (*Canis lupus familiaris*) (9%, 5/53) and gray fox (*Urocyon cinereoargenteus*) (7%, 4/53). It was impossible to amplify the mitochondrial D-loop housekeeping gene in nine samples (17%, 9/53), probably because of the presence of PCR inhibitors in feces, such as bile salts and complex polysaccharides [49]. An average of 96% to 99% molecular identity by BLAST search was found in the Genbank database corresponding to each species.

The phylogenetic tree was constructed using a dataset of Genbank sequences alignment with molecular D-loop fragment confirming *C. latrans* fecal samples from Costa Rica. The analysis revealed two different clusters; The first one comprises most of the samples gathered near the Irazú volcano (ACC), with one sample from the Horizontes sector (ACG) showing a branch support of 98. This cluster was closer to samples of *C. latrans* from Texas, Louisiana, Ohio, and Kentucky. In contrast, the other cluster consists of sequences

predominantly from ACG, and only one sequence from Irazú (ACC). Samples were closer to species registered in North Carolina, The Great Lakes, Wisconsin, and Canada (Figure 2).

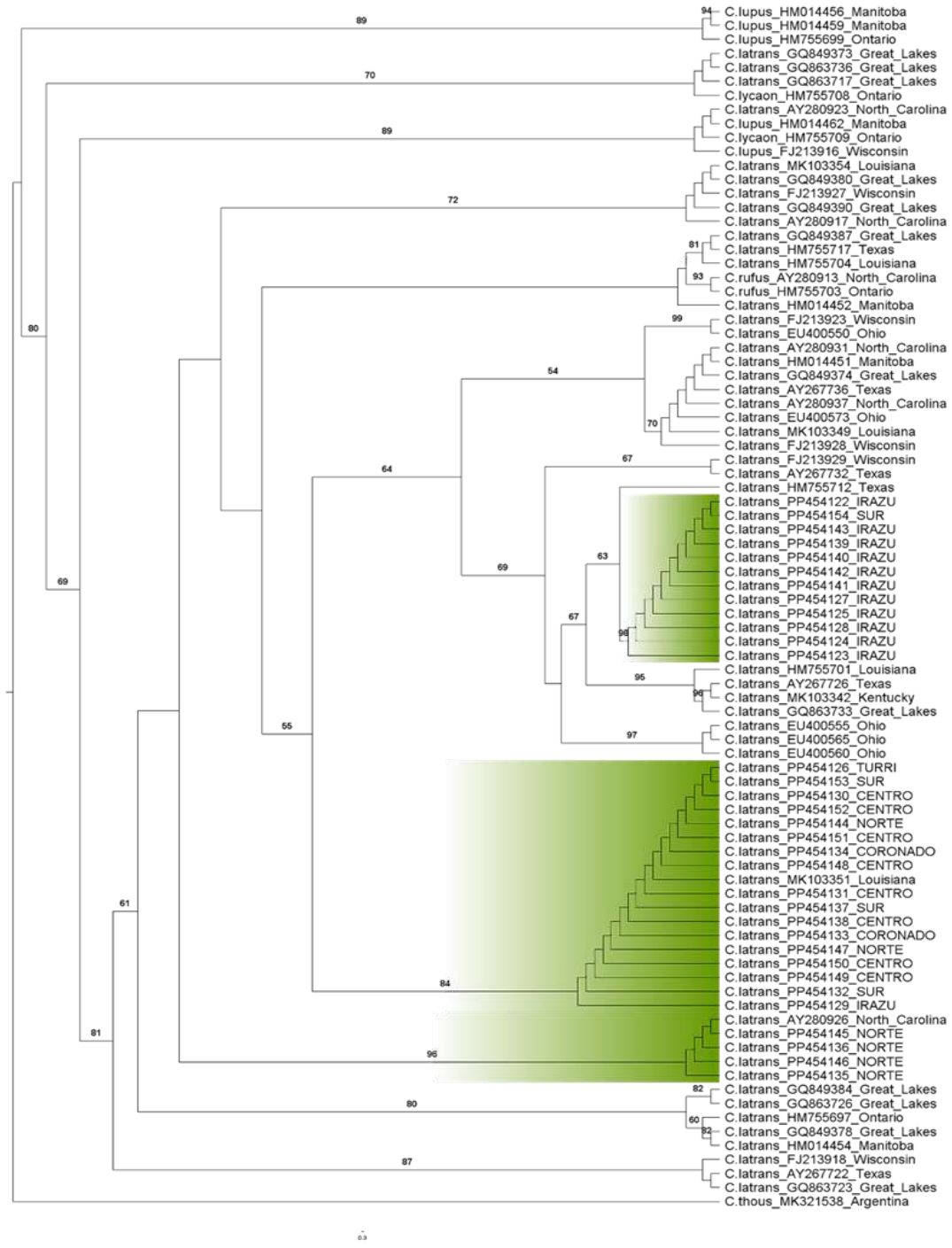

Figure 2. Phylogenetic tree of taxonomic placement based on mitochondrial D-loop fragment and maximum likelihood (ML) approach, confirming the molecular identity of the fecal DNA. Sequences from this study are identified in green color. *Cerdocyon thous* (MK321538) was used as an external group.

The genetic variation recorded in the 33 samples analyzed by the DNASP program showed that the two sampled habitats have h:3 haplotypes, with a haplotype diversity of Hd: 0.606, variance of 0.0024, and a nucleotide diversity of π 0.0099 (Table 1).

Table 1. List of the 33 individuals that support each mitochondrial D-loop fragment haplotype. The absolute frequency in the sample (Fr) and geographic distribution of haplotypes are also indicated. ACG: Guanacaste Conservation Area, and ACC: Central Conservation Area.

	Location	**Fr**	**Sample**
Haplotype 1	ACC	11	Irazú: M2, M3, M4, M5, M11, M12, M27, M28, M29, M30, M31.
	ACG	1	South: M56.
Haplotype 2	ACC	4	Turri: M8, M13, Iral: M17, M18.
	ACG	13	Center: M14, M15, M25, M45, M46, M47, M49, M54 South: M16, M24, M55, North: M36, M43.
Haplotype 3	ACG	4	North: M21, M23, M40, M41.

*3.3. **ARG detection in fecal samples of C. latrans***

PCR amplification of the seven ARGs evaluated showed that 97% of the assessed samples (n=35) amplified at least one of the ARGs analyzed. The highest prevalence by area corresponded to 78% for ACC and 54% for ACG. Regarding genes, the *bla*$_{TEM}$ gene corresponded to that with the highest presence (97%), while *catA*II was not found in any of the samples. The results are shown in Table 2 and 3. Table 2 states the individual results obtained.

Table 2. Endpoint PCR evaluation of seven antibiotic resistance genes in 35 fecal samples from coyotes (*C. latrans*) collected in this study.

Conservation Area	**Code**	***sul*I**	***catA*I**	***catA*II**	***bla*$_{TEM}$**	***tet*Y**	***tet*W**	***tet*Q**	**MDR[a]**
Central	M2	+	+	-	+	+	+	+	Yes
Central	M3	+	+	-	+	+*	+	+	Yes
Central	M4	+	+	-	+	+	+	+	Yes
Central	M5	+	+	-	+	+	+*	+	Yes
Central	M6	+	+	-	+	+*	+	+*	Yes

Central	M8	+	-	-	+*	+	+*	+	Yes
Central	M11	+	+*	-	+	+	+	+	Yes
Central	M12	+	+	-	+	+	+	+*	Yes
Central	M13	+	-	-	+	+	+	-	Yes
Central	M17	+	+*	-	+	+	+	+	Yes
Central	M18	+	-	-	+*	+*	+	+*	Yes
Central	M27	+	-	-	+*	+	+	+	Yes
Central	M28	+	-	-	+	+	+	-	Yes
Central	M29	+	+	-	+	+	+	+	Yes
Central	M30	+	+	-	+	+	+	+	Yes
Central	M31	+	+	-	+	+	+	-	Yes
Guanacaste	M14	+	+*	-	+	-	+	+	Yes
Guanacaste	M15	+	+	-	+	+	+	+	Yes
Guanacaste	M16	+	+*	-	+	+	+	+	Yes
Guanacaste	M21	+	+	-	+	+*	+	+	Yes
Guanacaste	M22	+	-	-	+*	+	+	-	Yes
Guanacaste	M23	+	+*	-	+	+	+	+*	Yes
Guanacaste	M24	-	-	-	+	-	+*	-	No
Guanacaste	M25	+	+	-	+	+	+	+*	Yes
Guanacaste	M36	+	-	-	+	+	+	+	Yes
Guanacaste	M40	-	-	-	-	-	-	-	No
Guanacaste	M41	+	-	-	+	-	+	-	Yes
Guanacaste	M43	+	-	-	+	-	+	-	Yes
Guanacaste	M45	+	-	-	+	-	+	-	Yes
Guanacaste	M46	+	-	-	+	-	+*	-	Yes
Guanacaste	M47	+	-	-	+	-	+	-	Yes
Guanacaste	M49	+	-	-	+	-	+	-	Yes
Guanacaste	M54	+	-	-	+*	-	+	-	Yes
Guanacaste	M55	+	+	-	+	-	-	-	No
Guanacaste	M56	+	-	-	+	-	-	-	No

*Samples analyzed with Sanger sequencing technology. [a] MDR: multi-drug resistance: samples resistant to three or more of the four classes of antibiotics tested, according to a previous classification reported [25,50].

Table 3. Prevalence of evaluated ARGs in the samples collected from the Guanacaste Conservation Area (ACG), and the Central Conservation Area (ACC) in Costa Rica.

Gene		**Sampled area**		**Prevalence**	
		ACG (n=19)	ACC (n=16)	n	%
***bla*TEM**		18	16	34	97%
***sul*I**		17	16	33	94%
***tet*W**		16	16	32	91%
***tet*Y**		7	16	23	66%
***tet*Q**		7	13	20	57%
***catA*I**		7	11	18	51%
***catA*II**		0	0	0	0%
Prevalence	n	72	88		
	%	54%	78%		

The taxonomic placement tree of the ARGs confirmed the identity and bacterial source of the sequenced genes. This process involved comparing the obtained sequences with reference sequences in GenBank, revealing a range identity of 79 to 100% with previously reported sequences. The tree illustrated the formation of distinct clusters for the six ARGs that underwent amplification. Genes *tet*W and *tet*Q shared the same ancestor with a branch support value of 60% and were subsequently divided into independent groups with bootstrap values of 81% and 99%, respectively. Finally, *tet*Y, *sul*I, and bla_{TEM} were grouped with a branch support value of 40%. The *tet*Y gene had an independent branch with a value of 100%, while bla_{TEM} had 98% support and *sul*I, 100%. The *catA*I gene was grouped in an independent cluster with a branch support value of 95%. For this gene, two samples (M14 and M16) from ACG formed another cluster within it, with a bootstrap value of 65% (Figure 3).

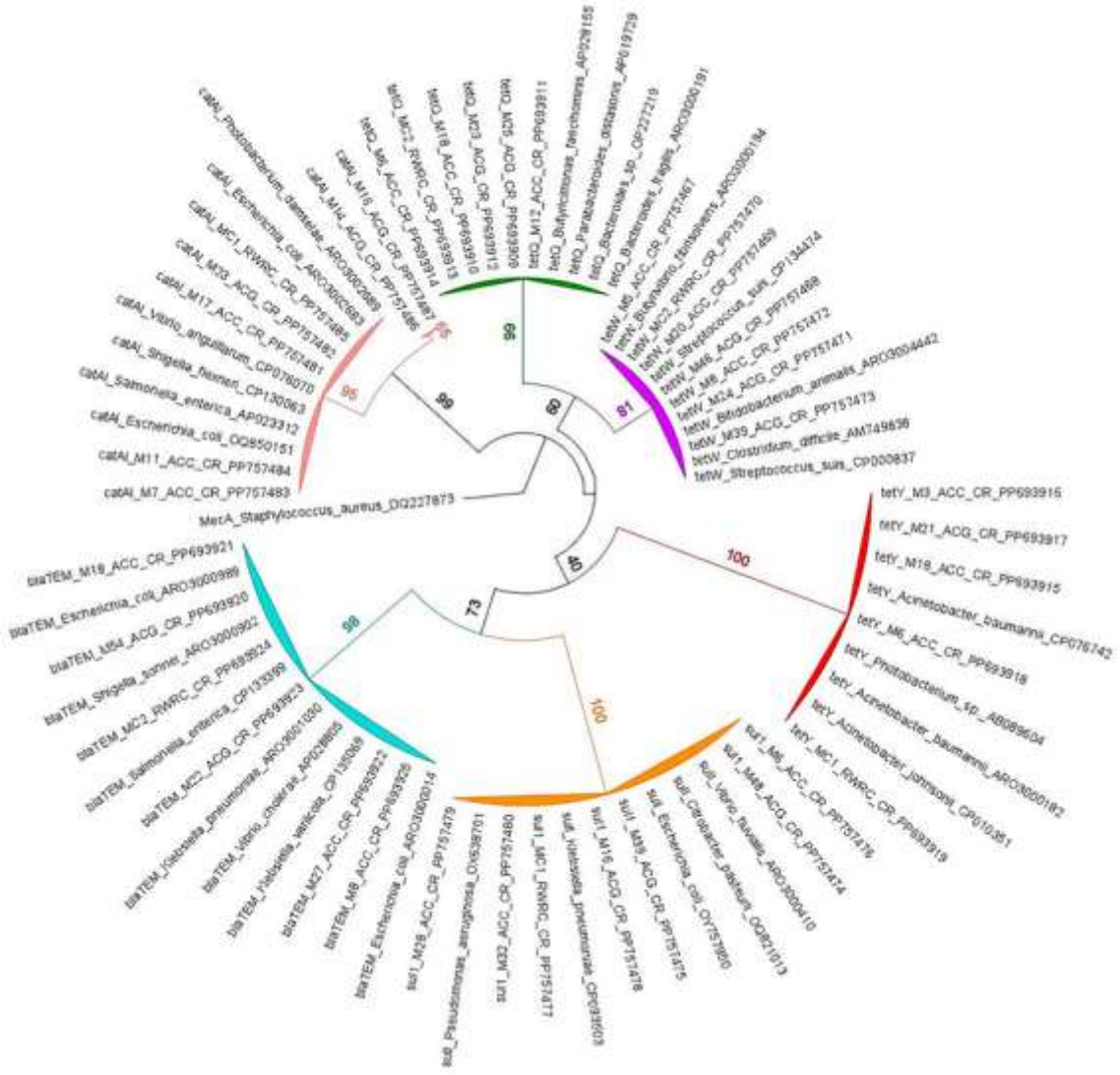

Figure 3. Maximum likelihood (ML) phylogenetic tree of taxonomic placement of the six detected ARGs in *C. latrans* fecal samples. Each color represents one of the ARGs analyzed (orange: *sulI*, pink: *cat*AI, blue: *bla*$_{\text{TEM}}$, green: *tet*Q, purple: *tet*W, and red: *tet*Y). Seventy sequences were used, represented in the 38 obtained in this analysis and accessed in the GenBank (PP693909 toPP693925, PP757467 to PP757487), complementing the 21 downloaded from the NCBI and 11 downloaded from The Comprehensive Antibiotic Resistance Database (CARD) [51] *Staphylococcus aureus* clone 249 *mec*A (DQ227873) as outgroup.

1. DISCUSSION

Antimicrobial resistance (AMR) is an emerging issue with significant public health implications [52]. However, its impact on natural ecosystems, particularly in protected areas, is not well understood, despite previous reports suggesting a link between its occurrence in wild bacterial populations and the level of human activity [18–20,53]. Hence, identifying a key sentinel species for environmental monitoring is crucial. This approach can provide scientific data that improves reports on habitat alterations due to anthropogenic activities. Such data would support the development of conservation plans for species and ecosystem protection measures from a conservation medicine perspective [50]. The coyote is a suitable candidate due to its ecology, meso-carnivore status, opportunistic feeding habits, wide territorial range, and high mobility. These traits allow coyotes to traverse various ecosystems, including urban, rural, and wild areas, making them ideal for environmental monitoring [54].

This study explores the potential of using coyotes as a sentinel species. This is contingent upon their colonization with microbes harboring ARGs, which could indicate previous exposure to antibiotics through contact with pharmaceutical residues from human sources, agricultural facilities, and contaminated food [16,55]. Once colonized, it is possible that coyotes could act as reservoir vectors, naturally amplifying the genes and, subsequently, transmitting them through horizontal gene transfer mechanisms such as conjugation, transformation, and transduction among bacteria of the same and different species [17,21,56–58].

Given the difficulties in capturing wild animals, it is necessary to adopt more passive methods for collecting environmental samples. Fecal samples and DNA, for example, are easier to obtain and do not require animal capture [59]. This approach also facilitates the characterization of their diet by identifying fruit residues, hair, bones, and seeds, which are consistent with the diverse diet documented in coyotes. As omnivorous generalist predators, coyotes adjust their foraging behavior to the available resources in their habitat, consuming a wide variety of foods such as rodents, lagomorphs, domestic mammals, birds, fruits, vegetables, arthropods, and ungulate carcasses. [60–62].

Regarding the genetic analysis carried out in this study, the amplification and sequencing of the mitochondrial D-loop region confirmed the identity of feces collected, of which 35 corresponded to *C. latrans*, a result consistent with the expected molecular weight of 360–364 bp described by De Barba et al. (2014) [39]. Samples corresponding to domestic dogs

(9%) exhibited a higher frequency in the ACC, potentially due to human activities surrounding this area, such as tourism and agriculture [63]. This confirms and validates the efficacy of the fecal collection methodology employed, ensuring the non-invasive collection of samples for *C. latrans*. This finding corroborates existing literature, underscoring the extensive distribution and adaptability of this meso-carnivore in Costa Rican ecosystems [1,2,4,64,65].

This study reveals, for the first time, the preliminary genetic variability of *C. latrans* populations from locations of Northern Costa Rica in ACG and ACC. The distributions of genetic variation observed in the phylogenetic tree analysis revealed the emergence of two distinct phylogenetic clusters between regions, suggesting a slight connectivity population by following the direction towards the south east. This discovery highlights the traits described in the literature, suggesting the coyote's capacity for rapid evolutionary adaptation, facilitating its survival and the inheritance of environmentally relevant genes [66]. Additionally, thanks to the national conservation areas and the protection of biologic corridors that promote structural and functional connectivity in the country, confirmation of adaptability across multiple studies in coyotes underscores the remarkable ecological versatility of this species [4,67–70]. These results corroborate considering coyotes as sentinels for the environmental status of the species, which is especially important in monitoring the AMR.

In genetic diversity evaluation, heterozygosity is generally beneficial since it allows more significant genetic variability and adaptability, leading to individuals with enhanced vigor, fertility, and resistance to diseases [71]. Our preliminary genetic analyses revealed a potential degree of consanguinity in the sampled population, which contrasts with the values previously reported in the Mexican population [72]. In that study, the authors analyzed 23 D-loop sequences, determining 13 haplotypes with haplotype diversity (Hd) of 0.945 and nucleotide diversity (π) of 0.0153. These results suggested a higher level of polymorphism in the Mexican population compared to our results in Costa Rica, potentially due to a bottleneck effect [46,73]. Additionally, the observed lower nucleotide diversity may indicate inbreeding in the sampled population, which could reduce biological fitness. Such inbreeding may harm the population, including increased susceptibility to diseases and the accumulation of environmental pollutants, such as ARGs [57,74].

It is important to note that 94% of the evaluated samples exhibited a multiresistant microbiome [25,50]. This finding is consistent with existing literature, which suggests a

direct correlation between proximity to anthropogenic areas and the presence of ARGs in the environment [15,17]. However, the current sample size is insufficient to draw definitive conclusions. The bla_{TEM} gene showed the highest number of amplifications, which may be correlated with the high selective pressure of beta-lactams in the country. Amoxicillin, reported as the most frequently used antibiotic due to its low price and broad spectrum, could be a driving factor [27]. This gene confers resistance to a broad range of drugs, covering three classes of antibiotics (carbapenems, cephalosporins, and penicillins), which are frequently prescribed globally for their effectiveness, broad-spectrum to address bacterial pathogens, and low toxicity in humans and animals [57,75]. The bla_{TEM} gene has also been detected in other Costa Rica wildlife, such as tapir (*Tapirus bairdii*) [29], and in surface water samples collected in areas inhabited by wild felines [31].

Tetracyclines are widely used across agricultural, human, medical, and veterinary domains and are notably recognized for their role as growth promoters in animal feed [19,54]. The genes evaluated in this study amplified more frequently in ACC, being present in almost all samples from this conservation area. This information opens the door to further investigation into the interface transmission of antibiotic resistance in ACC, highlighting the importance of closely monitoring this area. In this context, biodiversity conservation and ecosystem services are intertwined with human activities since nearly 60% of the national population lives near the Greater Metropolitan Area [76]. This situation is congruent with the significant risk for wildlife acquiring ARGs in proximity to human activities [77]. Previous studies have highlighted the widespread prevalence of tetracyclines in the country due to the extensive use of oxytetracycline [78]. This may be linked to the widespread dispersal of tetracycline resistance in the country, such as in crops [79], wild cats [18,23], tilapia tissues [80], and water systems [31,81], among others.

Sulfonamides are synthetic antibiotics commonly used to treat bacterial and protozoan infections. In this study, we only detected the *sul*I gene, which differs from results in other urban and rural environments in Costa Rica, where the presence of both genes in synanthropic animals and feces samples of felines has been reported [23]. A concerning finding from our research is that 51% of the samples amplified the *cat*AI gene with a high presence in both areas. The *cat*AI and *cat*AII genes were previously found in pigeons from public parks [25], which raises questions given the restricted use of chloramphenicol in production species in Costa Rica since 2021 [82]. This result could have potential implications for human health [83].

The phylogenetic tree for the six ARGs revealed differentiation processes in the analyzed sequences. The first evolutionary branching of 99 concerning the outgroup demonstrated that the *cat*AI gene did not present changes over time and was phylogenetically related. However, the findings in this cluster raised the interest in conducting a genetic lineage study to determine the cause of this internal branching supported by a bootstrap value of 65 for the samples collected from Guanacaste. The other ARGs showed a marked differentiation and speciation process. The *sul*I, *bla*$_{\text{TEM}}$, and *tet*Y genes exhibited a low branch support, likely related to the mechanisms of action. For example, the *sulI* gene modifies the antibiotic binding target site, while the *tetY* gene triggers an efflux mechanism to expel antibiotics from the cell. The *bla*$_{\text{TEM}}$ gene, in contrast, employs a mechanical resistance mechanism combined with enzymatic activity that inactivates the drug through acetylation or hydrolysis [84,85]. Our study also identified phylogenetic relationships between the *tet*W and *tet*Q genes, inhibiting protein synthesis at the 30S ribosomal subunit. [KBS1] This relationship, supported by a bootstrap mean value, was also observed in the study by Aminov et al., (2001) [86].

Previous studies in Costa Rica indicated that genes conferring resistance to tetracyclines, beta-lactamases, and sulfonamides are the most frequently detected across various environments, including wildlife, synanthropic animals, intensive crops, livestock, aquaculture farming, soil, water, and food sources [18,23,25–27,30,31,79,80]. Our findings are consistent with these results, showing that these ARG families were also the most prevalent in our study, though with a different order of prevalence: beta-lactamases, sulfonamides, and tetracyclines. Our data highlights the importance of evaluating each ecosystem individually, as the persistence of antimicrobial compounds and resistant bacteria may be influenced by factors such as antimicrobial use, host microbial communities, and geographic, environmental, and climatic conditions [87].

Our findings provide valuable insights for developing future strategies to mitigate antibiotic use and enhance environmental protection for wildlife. Our study establishes a baseline for monitoring the presence of ARGs in coyotes, using genetic data on population diversity and phylogeography to identify the status of populations. Therefore, continuous monitoring of genetic diversity and ARGs is crucial for assessing the effectiveness of conservation efforts and making necessary adjustments, emphasizing the importance of considering wildlife health from a conservation medicine perspective.

2. CONCLUSION

This study is the first report of ARGs in the ACG and ACC regions, and specifically in coyotes in Costa Rica. Our findings reveal the presence of multiresistant microbiomes, with a outstanding prevalence of genes encoding resistance to beta-lactamase genes and chloramphenicol. The ACC region exhibited the highest prevalence of ARGs, maybe due to its proximity to densely populated human areas. Moreover, this study presents the first population genetic data for coyotes in the country, revealing low but observed genetic differentiation between the two evaluated areas. This genetic differentiation is concerning given the potential impact on the health of the population and the risk of exposure to pollutants such as ARGs.

Key findings and implications from this study include:

· First Report of ARGs in coyotes: This is the first documented report on ARGs in coyotes in Costa Rica and Central America, revealing a significant diversity of these genes, especially against beta-lactamases.

· Mitochondrial DNA sequencing: For the first time, we utilized D-loop sequences of mitochondrial DNA to establish maternal lineages of coyote populations in Costa Rica.

· Sentinel species: *Canis latrans* can serve as an effective sentinel species for monitoring the interface between human and natural environments. Monitoring ARGs in areas undergoing land use changes is crucial, as these regions often show increased antimicrobial resistance gene diversity.

· Genetic diversity and connectivity: The observed genetic diversity suggests some level of population connectivity. However, further studies with expanded sampling are needed to validate these results and assess potential genetic drift in coyote populations.

· Impact of habitat destruction: Habitat destruction, reduced food availability, and environmental contaminants, including ARGs, can negatively affect coyote health and biological fitness.

· Antibiotic resistance gene prevalence: A high percentage of samples exhibited multiple antibiotic resistance, with the highest prevalence in the ACC region. To promote the responsible use of pharmaceutical medications in humans, veterinarian care, and agriculture, it is advisable to implement socialization programs in this area.

· Resistance to phenicols: Given that chloramphenicol is banned in animals, it is urgent to identify how *catA*I can be found in the environment.

3. DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

4. DATA AVAILABILITY

Data will be made available on request.

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6. CRediT authorship contribution statement

Lina Puentes-Sánchez: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Rodolfo Umaña-Castro**: Writing – original draft, Visualization, Data curation, **Kinndle Blanco-Peña**: Writing – original draft, review & editing, Funding acquisition, **Carolina Sáenz-Bolaños**: Writing – review & editing, Project administration, **Victor Montalvo-Guadamuz**: Writing – review & editing, **Kevin Lloyd-Alcock**: Writing – review & editing, **Isabel Hagnauer-Barrantes**: Writing – review & editing, **Kari Brossard Stoos**: Writing – review & editing, **Carolina Sancho-Blanco**: Writing – original draft, review & editing, Formal analysis, Data curation, Conceptualization, Funding acquisition

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Artículo II: Antimicrobial Resistance in Gray Fox *Urocyon cinereoargenteus* in the Área de Conservación de Guanacaste, Costa Rica.

Sustentante

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Antimicrobial Resistance in Gray Fox *Urocyon cinereoargenteus* in the Área de Conservación de Guanacaste, Costa Rica.

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ABSTRACT:

This study aimed to assess antibiotic resistance in gray foxes (*Urocyon cinereoargenteus*) in the Área de Conservación de Guanacaste, Costa Rica. We evaluated the presence of seven antibiotic resistance genes (ARG: *sul*I, *cat*AI, *cat*AII, bla_{TEM}, *tet*Q, *tet*W, and *tet*Y) in three fecal samples from free-ranging *U. cinereoargenteus* using PCR assays. Of the seven antibiotic resistance genes assessed, *tet*W was found in all samples, whereas *cat*AI was not detected. Multidrug resistance was determined in all samples (one or more ARG were detected in all samples). Our findings confirm that wild animals could potentially be bioindicators, reservoirs, or carriers of antibiotic-resistant bacteria in the environment, with *U. cinereoargenteus as a* representative sentinel in external border sectors of conservation areas.

Keywords: Antibiotic resistance genes, environmental pollution, PCR, wildlife.

The gray fox (*Urocyon cinereoargenteus* Schreber, 1775) is a canid species categorized as "Least Concern" on the IUCN Red List (Roemer et al. 2016) and has a lifespan of 10 to 15

years. Its home range varies according to the gender and landscape resources, with males occupying areas between 135 and 140 ha and females from 105 to 110 ha. This solitary mesopredator is known to occasionally share space with three or five other individuals within 2 km^2 of land (200ha) (Santamaría et al. 2009). The gray fox inhabits caves and is considered an opportunistic small carnivore (1.8-3.5 kg) with an omnivorous diet behavior (Carrillo et al. 2002). It is also a significant seed disperser in the ecosystems, depending on food availability and the year's season. These animals are nocturnal or crepuscular, but diurnal activities are also common. It is distributed from Canada to Northern South America, covering various habitats, including tropical forests, temperate deciduous forests, and scrublands with a high density of shrubs, as well as in urban and rural areas (Gallina et al. 2016). The gray fox is the only canid with the ability to climb trees due to its semi-retractable claws.

In Costa Rica, this species has been reported in low and middle lands of the Pacific slope up to 2600 m.a.s.l. (Carrillo et al. 2002). One of the ecosystems where gray foxes are located is the dry forest in the Área de Conservación Guanacaste (ACG). Historically, this land has been transformed for agroindustry purposes; however, it has been under forest restoration since the establishment of Project Parque Nacional Guanacaste (Lobo 2016). The biodiversity in this area is adapted to alternating dry and rainy seasons. The biological reserve spans from the Pacific Ocean, through dry forested lowlands, over the Volcanic Guanacaste Mountain Range, and down into the rain-forested Caribbean lowlands, providing necessary resources for multiple mammal species (Janzen and Hallwachs 2020).

Compared to other canids, gray foxes have a broader food niche (Gallina et al. 2016), which may contribute to their potential infection with antimicrobial resistance (AMR) (Vittecoq et al. 2016). AMR is considered one of the many factors contributing to environmental pollution, with major human sources including landfills, inadequately treated wastewater discharged into rivers and lakes, and waste from intensively managed livestock farms (Swift et al. 2019). Additionally, the presence of antibiotic resistance genes (ARGs) can cause microbiota imbalances, negatively impacting the host's health and thereby, affecting their ecological role (Lee et al. 2022). This study aimed to evaluate whether fecal samples of gray foxes collected in ACG showed the presence of ARGs.

Three fecal samples of *U. cinereoargenteus* were opportunistically collected from May to August 2022. The collection points were located on roadsides or sidewalk areas in external border sectors of Santa Rosa and Pocosol, zones managed by the government of Costa Rica through the National System of Conservation Areas (SINAC). The collection process was carried out according to the entry and collection permits obtained for the conservation areas (R-CM-UNA-004-2022-OT-CONAGEBIO). Samples were stored individually in 50 mL conical tubes labeled with identification (ID), date, and GPS coordinates. The samples were then transported to the Genomic Analysis Laboratory (LAGen) of the School of Biological Sciences, at the Universidad Nacional while maintaining the cold chain at 4°C. DNA extraction was performed with the QIAamp™ DNA Stool Kit Handbook, following the manufacturer's instructions. The concentration of extracted DNA was determined through a UV-visible microvolume spectrophotometer, and the purity of the nucleic acids was evaluated by the absorbance coefficients A260/A280 and A260/A230 (NanoDrop 2000, Thermo Fisher Scientific Inc., Waltham, MA, USA). Finally, the integrity of the DNA was determined by gel electrophoresis.

Endpoint PCR was used for the molecular identification of gray fox feces, presence of bacteria, and detection of ARG. First, *U. cinereoargenteus* identity was determined by amplifying a short segment of the mtDNA control region (D-loop) fragment through the primers SIDL 5-TCTATTTAAACTATTCCCTGG-3 and H3R 5-CCTGAAGTAGGAACCAGATG-3 (De Barba et al. 2014). Subsequently, it was corroborated through Sanger sequencing in two directions using a 3500 genetic analyzer (Applied Biosystems®) by BigDye™ Terminator V3.1 Kit, following the manufacturer's instructions. The sequences obtained were edited in the Geneious Prime® program v.2023.2.1 (Biomatters Ltda®), and then analyzed with the online program BLASTn, available at the National Center for Biotechnology Information (NCBI), to compare the sequences obtained with the GenBank database (National Center for Biotechnology Information 2024) and confirm the identity of each sample.

A taxonomic placement analysis was carried out, complemented with sequences from the NCBI, and red fox (*Vulpes vulpes* [KJ846639]) as an outgroup. Sequence matrices were initially created by aligning them with MAFFT using the Geneious Prime® program

(v.2023.2.1, Biomatters Ltda). To construct the taxonomic tree, IQ-TREE (https://www.hiv.lanl.gov/content/sequence/IQTREE/iqtree.html) multicore program was used. Phylogenetic topology was constructed using the maximum likelihood (ML) algorithm. The model-selection method to determine the best model was HKY+F+G4 (AICc= 4772.596). The parameters used for phylogenetic inference were the free rate heterogeneity and node support calculation derived from the bootstrap method with 10,000 replicates. Finally, the tree obtained was visualized and edited with the FigTree v1.4.4 program.

We detected the presence of bacterial DNA by amplifying the 16S rDNA gene, using the primers 27F and 1492R (Chen et al. 2015). The amplification was considered positive for bacteria if the amplicon size was between 251 and 299 bp. PCR reactions were performed to detect the genes encoding resistance to sulfonamides (*sul*I) (Yuan et al. 2021), phenicols (*cat*AI, *cat*AII) (Sacristán et al. 2020), beta-lactams (bla_{TEM}) (Kozak et al. 2009), and tetracyclines (*tet*Q, *tet*W, and *tet*Y) (Sacristán et al. 2020). For each reaction, we used 25µL of the reaction mixture containing 2.5µL of the 2/8 dilution of DNA template, 12.5µL of 2X Dream Taq PCR Master Mix (Thermo Fisher Scientific Inc.), and 0.8µL of each primer at a concentration of 10µM and 8.4µL of ddH_2O. The primer extension previously described was carried out with the thermal profile standardized as follows: Denaturation: 1 cycle (95°C x 1 min), annealing: 36 cycles [95°C x 15s, 60°C* x 45s (*only bla_{TEM} at 55°C), 72°C x 45 s], and elongation of 1 cycle (72°C x 10 min).

We confirmed the species through taxonomic placement analysis of D-loop. The phylogenetic tree exposed one independent cluster for the *Urocyon* species compared to the other wild canid species present in Costa Rica. The *Urocyon* cluster exhibited the gray fox lineage nested with sequences of U. *littoralis*. Similar findings have been reported in genomic assessments of the *Urocyon* species. Through mitochondrial haplotype data, these findings suggest cryptic divergence patterns in the 16 subspecies described. Additionally, a strong divergence was evidenced between eastern and western lineages in North America, which could be caused by ancient isolation during the mid-Pleistocene (Armstrong et al. 2024). After confirming the species, the three sequences were accessed in GenBank with the records PP974326, PP974327, and PP974329 (Fig 1).

We identified at least one ARG in each sample. The genes *tet*W, *sul*I, and *bla*$_{TEM}$ were amplified in all samples. The other genes encoding resistance to tetracyclines, *tet*Q, and *tet*Y, were present in two samples. Finally, from the phenicol's genes analyzed, *cat*AI was detected only in one sample. According to a previous classification reported, multi-drug resistance was determined in all samples (Swift et al. 2019) (Table 1).

Our findings evidenced the prevalence of antibiotic-resistant bacteria in wildlife, which could be influenced by various factors, including the habitat use and foraging behavior of the species studied, especially with human impacts on the environment (Ramey and Ahlstrom 2020). However, it is necessary to address these areas with direct control strategies aiming at this public health concern.

To our knowledge, this is the first report of ARGs in gray foxes in Costa Rica. However, a previous study in foxes (*Lycalopex culpaeus*) inhabiting anthropized landscapes in central Chile obtained similar results to ours, finding comparable multi-drug resistance in their samples (Cevidanes et al. 2020). Studies in feline populations in other protected areas from Costa Rica showed a frequency of 50% in ARGs to tetracyclines, sulfonamides, and cephalosporins, in concordance with our study. The gene *cat*AII was not detected in any of the samples; however, Angulo et al. (2023), identified this ARG in wild cats in Costa Rica.

In the case of *bla*$_{TEM,}$ this is one of the genes with more monitoring frequency in wildlife since cephalosporins are antibiotics of critical importance to human health. Our analyses exposed the presence of this gene in the samples. Similar results were reported by other studies of wildlife in Costa Rica, which found high resistance to the antibiotic cephalosporin in gram-negative cells of monkey's oral cavity (Rodríguez-Rodríguez et al. 2007). This gene was the most frequently amplified in water surface samples collected in Costa Rican habitats with wild feline presence in two national parks and their surrounding areas (Vargas-Villalobos et al. 2024).

Another factor considered in our results was the border location of the protected areas when the collection points were found. Those points were near human activities, which represents the risk of having a horizontal transfer of resistance genes. In this case, our results correlate with a previous study in which omnivore animals frequently feed on carcasses and live close

to humans and domestic animals, which may increase the risk of AMR transmission (Bamunusinghage et al. 2022). The risk of catching ARG from wildlife tends to increase when such species are exposed to anthropogenic sources of AMR, such as wastewater or landfills. A study in the Greater Metropolitan Area of Costa Rica exposed the presence of genes encoding resistance to sulphonamides and cephalosporines (Rivera-Montero et al. 2023). The authors described the same genes found in our research, showing the direct and indirect interaction between humans and wildlife (Torres et al. 2021).

Our findings reveal, for the first time, the antibiotic resistance in gray foxes (*U. cinereoargenteus*) in Costa Rica, a country recognized for continually seeking to know the dynamics of dissemination of this resistance in different ecosystems. Monitoring of ARGs should continue to understand their development across various ecosystems to help focus programs to educate the population and control the products that could emphasize this resistance according to the requirements of each zone.

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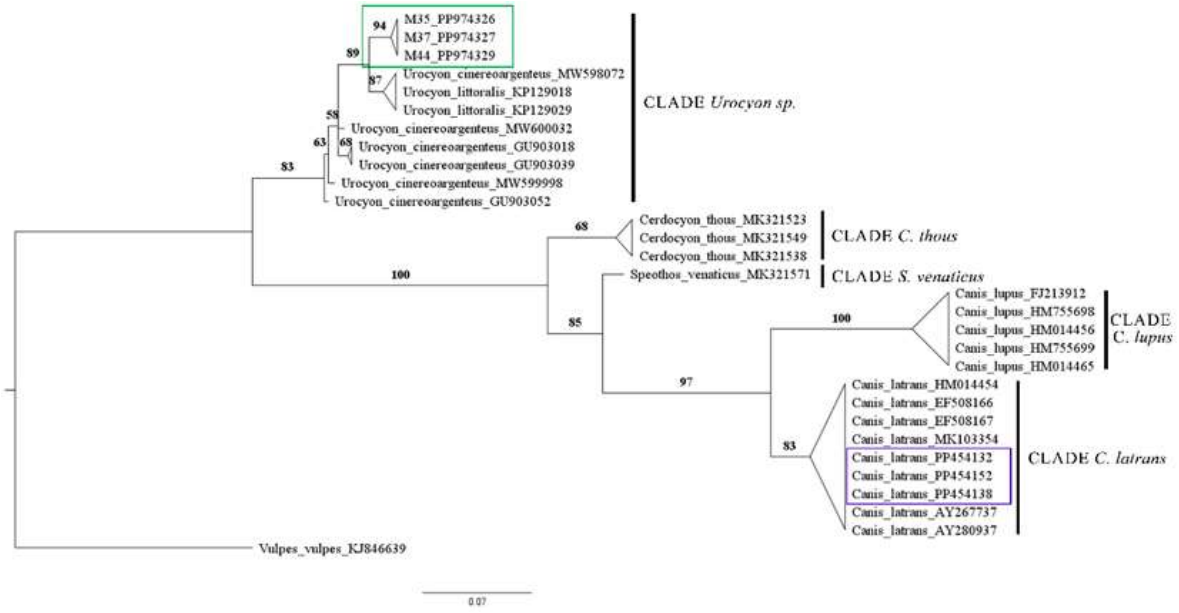
Table 1. Antimicrobial resistance of gray fox (*U. cinereoargenteus*) fecal samples from Área de Conservación Guanacaste (ACG) collected from May to August 2022.

Sample code	*Sul*I	*cat*AI	*cat*AII	*bla*$_{TEM}$	*tet*Y	*tet*W	*tet*Q	MDR[a]
Z1	+	-	-	+	+	+	+	Yes

Z2	+	+	-	+	+	+	+	Yes
Z3	+	-	-	+	-	+	-	Yes
Prevalence (%)	100%	34%	0	100%	67%	100%	67%	

[a] MDR: multi-drug resistance: samples to have resistance to three or more of the four classes of antibiotics tested, according to a previous classification reported (Swift et al. 2019).

Figure 1. Taxonomic placement analysis of three sequences of D-loop obtained from fecal DNA samples from Área de Conservación Guanacaste (ACG) collected from May to August 2022. Sequences from this study are identified in green. *Canis latrans* sequences from Costa Rica are identified in blue.



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Artículo III: **Prevalence of Antibiotic Resistance Genes in coyotes (*Canis latrans*) inhabiting two conservation areas with border anthropized landscapes in Costa Rica.**

Sustentante

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Manuscrito en revisión para envío a revista de pares

Prevalence of Antibiotic Resistance Genes in coyotes *(Canis latrans)* inhabiting two conservation areas with border anthropized landscapes in Costa Rica.

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Abstract

The extensive utilization of antibiotics in human, animal, and plant health has resulted in the pervasive contamination of the environment due to the misuse and incomplete metabolism of these pharmaceuticals. This has resulted in the emergence of antibiotic-resistant bacteria as a consequence of selective pressure. The transfer of antimicrobial resistance genes (ARGs) via reservoirs in the environment is being facilitated by human activities, thereby exacerbating the situation. The objective of this study was to quantify the prevalence of nine ARGs in coyotes (*Canis latrans*) feces and analyzed the results with spatial analysis to look for possible correlations between our finds of ARGs and geographic characteristics in two protected areas with anthropized landscapes in Costa Rica. Scat samples were collected in the Central Conservation Area (CCA) and Guanacaste Conservation Area (GCA) between March and August 2022 through the use of non-invasive methods. The samples were subjected to DNA extraction. The presence of nine ARGs was determined through quantitative PCR (qPCR). The ARGs included in the study were sulfonamides (*sul*I, *sulI*I), phenicols (*cat*AI, *cat*AII), beta-lactams (bla_{TEM}), tetracyclines (*tet*Q, *tet*W, *tet*Y), and

quinolones (*qnr*S). All samples exhibited amplification of at least one ARG. Four samples (11%) demonstrated amplification of all nine genes. A total of (74%) of the samples (26/35) exhibited a multidrug-resistant microbiome. With the most important resistance genes (*sul*II, *tet*Q, *tet*Y and *catA*I), it was observed that there is a difference in the number of genes registered per conservation area and between the same genes (Permanova, F= 3.944; p= 0.0084). *catA*II, shows the lowest abundance. Meanwhile, *sul*II and *tet*Y genes have differences in their concentration between both conservation areas (Dunnpost huc, $p< 0.05$). The canonical correspondence analysis, conducted with the geographic information system data, revealed that the majority of ARGs are correlated with roads. However, the blaTEM gene demonstrated a particularly strong correlation with cattle and pig farms. These findings have implications for environmental health and wildlife management to mitigate the dissemination of antimicrobial resistance, the data underscore the necessity for sustained surveillance and targeted interventions.

Introduction

The advent of antibiotic drugs constituted a highly significant development in the field of medicine, offering a powerful tool for combating infections. Their applications are diverse, encompassing human, animal, and crop health (Richardson, 2017). The imprudent use of antibiotics and the incomplete metabolization of these drugs after administration have resulted in the excretion of their metabolites in an unaltered state via urine and feces (Truong et al., 2021). These metabolites permeate a variety of environments, exerting persistent selective pressure on the microorganisms that inhabit these ecosystems (Czatzkowska et al., 2022). The selective pressure exerted on bacteria in the presence of antibiotics can result in the emergence of antibiotic-resistant strains (Skandalis et al., 2021).

The activities of humans have resulted in the promotion of a diverse pool of antimicrobial resistance genes (ARGs) that are capable of rapid transfer between different environmental reservoirs, including soil, water, and wildlife (Zhuang et al., 2021). This propagation facilitates the accumulation of these ARGs in pathogenic and non-pathogenic bacteria, thereby contributing to the formation of the resistome. The fitness costs associated with maintaining ARGs in bacteria are relatively low, contributing to the perpetuation of the resistome over time (D'Costa et al., 2011; Zhuang et al., 2021).

The discovery of antibiotic resistance genes has been extensively documented in a multitude of wildlife species across diverse ecosystems and continents (Torres et al., 2021). The prevalence of ARGs is increasing in wildlife populations situated in close proximity to human-inhabited areas. This phenomenon facilitates the large-scale dissemination of these genes through migratory processes (Vittecoq et al., 2016; Worsley-Tonks et al., 2021). The potential for this resistance to spread through the food chain is significant, occurring either indirectly through the consumption of bacterial flora present in prey or directly from fruits, leaves, or water that comprise the diet of the evaluated individuals (O'mahony et al., 2006). These findings have also demonstrated a direct impact on ecosystem health, as wildlife with greater reliance on human resources have exhibited alterations in their role within the food chain, leading to an increased dependence on low-quality diets that contribute to immunosuppression, alterations in the nutrient flow of the biological community, and an increased predisposition to disease acquisition (Di Bitetti, 2008; Murray et al., 2015).

A number of studies have demonstrated that wildlife can be employed as indicators of antibiotic resistance in the environment. This is due to the fact that monitoring wildlife provides information on the spatial and temporal occurrence of resistance genes (Cevidanes et al., 2020; Dias et al., 2022; Jarma et al., 2021; E. Miller et al., 2020; Nieto-Claudin et al., 2019; Plaza-Rodríguez et al., 2020; Sacristán et al., 2020; Wang et al., 2022; Worsley-Tonks et al., 2020). Consequently, geographic information systems (GIS) have become a widely utilized tool in the medical field. GIS is an invaluable tool for deepening our understanding of the ways in which humans interact with their environment (de la Torre et al., 2012). It is particularly effective for geographically linking data to potential sources of environmental exposures and the locations of health resources (Hewapathirana & Wijayarathna, 2010). The application of spatial analysis has been pivotal in examining the geographical variations in the occurrence and extent of antimicrobial resistance (ARM) contamination (Jones et al., 2008).

In this study, we sought to characterize the prevalence and spatial variation of antibiotic genes, namely sulfonamide (*sul*I and *sulII*), phenicol (*cat*AI, *cat*AII), beta-lactam (bla_{TEM}), tetracycline (*tet*Q, *tet*W, and *tet*Y), and quinolone (*qnr*S), in coyote scat in two conservation areas with different borderline intensive urban and agricultural activity. The objective was to

determine whether the presence and quantity of ARG found varies according to human activities or geographic factors, through GIS analysis to provide information for monitoring and management purposes against AMR.

Material and methods

Study area

Samples were obtained from two conservation areas under the purview of the Costa Rican government through the National System of Conservation Areas (SINAC). The Central Conservation Area (CCA), which encompasses an area of 650,918 hectares, comprises the La Selva Biological Station, the Alberto Manuel Brenes Biological Reserve, Braulio Carrillo National Park, Quetzales National Park, and the Cordillera Volcánica Central Forest Reserve. The region is distinguished by elevated topography, dense forest cover, and a network of rivers, in addition to extensive livestock farming (Angulo et al., 2023; Chassot et al., 2009; SINAC, 2019). The second area under consideration is the Guanacaste Conservation Area (ACG), which is situated to the northwest of Costa Rica. The area in question encompasses 163,000 hectares. The following protected areas were included in the study: The protected areas included in the study were Santa Rosa National Park, Guanacaste National Park, Rincón de la Vieja National Park, Junquillal National Wildlife Refuge, and Horizontes Experimental Forest Station. The region encompasses a range of ecosystems representative of tropical climates, including coral reefs, dry forest, tropical forest, cloud forest, and rain forest (Janzen & Hallwachs, 2020; Lloyd-Alcock, 2020; SINAC, 2019). These areas have been subjected to human activities, including the intensive utilization of agrochemicals and fertilizers, the diversion of river courses, and the contamination of water sources (Costa Rica). (Costa Rica. Ministerio de Ambiente y Energía, 2001; Instituto de Investigaciones Marinas y Costeras, 2001). These variables permit an investigation of the relationship between the presence of antibiotic resistance genes (ARGs) and the proximity of human activities via the niche of the mesopredator coyote.

Coyote scat samples

Canid scat samples were collected opportunistically by noninvasive methods (i.e., without capturing the animals) between March and August 2022. This research was conducted in collaboration with researchers from the International Institute for Wildlife Conservation and Management (ICOMVIS). The field collector recorded the species identification based on color, scat size, and tracks. The samples were stored individually in 50 mL conical tubes, which were appropriately labeled with the relevant identification (ID), date, and GPS coordinates, and the cold chain was maintained at four degrees Celsius. Subsequently, the samples were transported to the Regional Institute for Studies in Toxic Substances (IRET) for storage at -80°C. The species identification was conducted at the School's Genomic Analysis Laboratory (LAGen) of Biological Sciences, Universidad Nacional. A species-specific primer was used to amplify a short segment of the mtDNA control region (D-loop) fragment through the primers SIDL 5-TCTATTTAAACTATTCCCTGG-3 and H3R 5-CCTGAAGTAGGAACCAGATG-3 (De Barba et al., 2014). Sequences accessed in GenBank with the records (PP454122-PP454156). All samples were collected and analyzed according to Costa Rican regulations (permits R-CM-UNA-004-2022 -OT-CONAGEBIO).

Antibiotic resistance genes (ARGs) detection

DNA was extracted directly from all fecal samples (180–220 mg) using the QIAamp™ DNA Stool Kit (Qiagen), following the manufacturer's instructions. ARGs were screened by performing quantitative PCR (qPCR) using a CFX96 Real-Time System (Bio-Rad, USA). The reaction mixture consisted of 5 µL of PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific), 0.4 µL of each forward and reverse primer (10 µM) (Invitrogen), 0.25 µL of Yellow Sample Buffer (Thermo Fisher Scientific), 2.95 µL of RNase-free water, and 1 µL of template DNA, for a final volume of 10 µL. Negative control samples were treated similarly with the exclusion of template DNA. The 16S rRNA gene was amplified to confirm the presence of bacterial genetic material in each sample. Samples were considered as validated when a ten-fold dilution showed a cycle threshold (Ct) less than 25 (Esperón et al., 2020). Once validated the present of DNA bacterial, we analyzed samples by a panel of up to nine different ARGs encoding resistance to five different antimicrobial classes as representatives of the main antimicrobials generally used in veterinary and human medicine: tetracyclines (*tet*W, *tet*Q and, *tet*Y), sulfonamides (*sul*I and *sul*II), phenicols (*cat*AI and

*catA*II), quinolones (*qnr*S) and betalactams (*bla*$_{TEM}$) The specific primers and amplification conditions for each ARG are detailed in Table 1 and 2.

Table 1. Gene primers used for detection of AGRs in samples of DNA.

Target gen	Primer sequences (5′→3′)	Amplicon length (bp)	Annealing temp (°C)	Reference
16s rRNA	515F-TCGAAGTATGGTAATTGTGTGYCAGCMGCCGCGGTAA	291-300	50	(Caporaso et al., 2011; Parada et al., 2016)
	806R-AGTCAGCCAGCCGGACTACNVGGGTWTCTAAT			
bla$_{TEM}$	F-CACTATTCTCAGAATGACTTGGT	85	60	(Lachmayr et al., 2009)
	R-TGCATAATTCTCTTACTGTCATG			
cat I	F-GGTGATATGGGATAGTGTT	349	60	(Yoo et al., 2003)
	R-CCATCACATACTGCATGATG			
cat II	F-GATTGACCTGAATACCTGGAA	567	63	(Yoo et al., 2003)
	R-CCATCACATACTGCATGATG			
qnrS	F-GACGTGCTAACTTGCGTGAT	300	60	(Marti & Balcázar, 2013)
	R-TGGCATTGTTGGAAACTTG			
sul I	F-CACCGGAAACATCGCTGCA	158	63	(Luo et al., 2010)
	R-AAGTTCCGCCGCAAGGCT			
sul II	F-CTCCGATGGAGGCCGGTAT	190	60	(Luo et al., 2010)
	R-GGGAATGCCATCTGCCTTGA			
tetY	F-ATTTGTACCGGCAGAGCAAAC R-GGCGCTGCCGCCATTATGC	181	63	(Aminov et al., 2002)
tetQ	F-AGAATCTGCTGTTTGCCAGTG	169	63	(Aminov et al., 2002)
	R-CGGAGTGTCAATGATATTGCA			
tetW	F-GAGAGCCTGCTATATGCCAGC	168	63	(Aminov et al., 2002)
	R-GGGCGTATCCACAATGTTAAC			

Table 2. Thermal protocols used for the qPCRs.

Target gen (s)	Initial denaturation	Amplification cycling	Melting curve
16s rRNA	94.0°C 10min	35 cycles of 94.0°C 45s, 50.0°C 1min, 72.0°C 1.30min and 72.0°C 10min	50.0C° to 95.0C° increment 0.5C° for 5s
*catA*II, *sul*I, *tet*Q, *tet*W, *tet*Y	98.0°C 3min	35 cycles of 98.0°C 15s, 63.0°C 30s, 72.0°C 1min and 72.0°C 10min	65.0C° to 95.0C° increment 0.5C° for 5s
bla_{TEM}, *catA*I, *qnr*S, *sul*II	50.0°C 2min, 95.0°C 2min	35 cycles of 95.0°C 10s, 60.0°C 30s and 95.0°C 15s	60.0C° to 94.5C° increment 0.3C° for 1min

The CT values within the 12 to 30 range were considered valid; samples exceeding 30 but below 34 underwent sequencing (Macrogen, Seoul, South Korea) to confirm gene presence. The sequences obtained were edited in the Geneious Prime® program (v.2023.2.1, Biomatters Ltda), then analyzed with the online program BLASTn (Altschul et al., 1990) available at the National Center for Biotechnology Information (NCBI), to compare the sequences obtained with the GenBank database and The Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023) thus validated the identity of the results. We approximated the number of ARG copies we performed for triplicate each analysis, where the average was used in the formula (Nieto-Claudin et al., 2019):

$$\text{Log10 } (\textit{percentage of an ARG} = 2 + 0.33 \times (Ct_{16SrRNA} - Ct_{ARG})$$

$Ct_{16SrRNA}$ was the cycle threshold for bacterial determination, Ct_{ARG} was the cycle threshold for each gene, and 0.33 was the mean slope for all the genes tested. We expressed results in Log10 of the hypothetical percentage of bacteria that each gene presents for the percent load of ARG. Because the exact date the coyotes defecated the samples is unknown, this formula allows a relative quantification based on the number of bacteria in each sample. Samples were also classified into "multiresistant microbiome" defined as at least three ARGs encoding resistance to different groups of antimicrobials and "non-multiresistant microbiome" (Angulo et al., 2023; Blanco-Peña et al., 2017; Cevidanes et al., 2020; Swift et al., 2019).

Geographic analyses

We also considered the geographic and physical characteristics of the areas. The coyote home range was defined as a 37 km² diameter around each collection site, as specified by the Universidad Nacional, Costa Rica, through the FIDA project, "Comprehensive approach to the situation of the coyote (*C. latrans*): Local perception and preliminary analysis of its ecology in two regions with different socioeconomic activities in Costa Rica" (0193-21). Were identified livestock farms (pig, cattle, and poultry), roads, rivers, forest and remaining vegetation index (IVR) as potential contact sources within each area. To determine the spatial distribution of the relationship between the ARG prevalence and potential contact sources was analyzed used ArcGIS geographic information system software (version 10.3; Esri, Redlands, California, USA), incorporating layers such as geographic data for Costa Rica, protected areas, biological corridors, forest cover, topographic relief, rivers (1:200,000 scale), villages, and land use (Ortiz-Malavasi, 2014). Data related to livestock production was generously provided by the National Animal Health Service (SENASA, Heredia, Costa Rica) from a census performed in 2014.

Statistical analyses

The values of each resistance gene were transformed into a natural log due to the presence of skewed distributions. Despite this transformation, the variables did not yet exhibit a normal distribution. To identify the most prevalent and representative resistance genes, a heat map was constructed. To ascertain the relationship between the quantitative ARGs behavioral observation data and geographic characteristics, a canonical correspondence analysis was conducted. Subsequently, a principal component analysis was conducted for both processes to ascertain which genes had the greatest influence and to avoid correlations between them. Furthermore, a PERMANOVA (Permutational Multivariate Analysis of Variance) was performed to determine whether there were differences in the concentrations of resistance genes between and within groups, with the latter comparison being between genes within the same conservation area. All analyses and graphics were conducted using the R programming language, version 4.3.2 (R Core Team, 2023), in addition to the ggplot2 package, version 3.4.4 (Wickham, 2016), and the ggdist package, version 3.3.2) (Kay, 2024).

Results

All samples demonstrated amplification of at least one of the nine genes that were subjected to evaluation. Four samples (11%) demonstrated amplification of all evaluated genes. A total of 74% of the samples exhibited a multidrug-resistant microbiome, with 62% positive in ACC, and 84% in ACG. All samples tested positive for the genes *tet*W, *tet*Q, and *sul*I. The gene *tet*Q exhibited the highest prevalence of amplification (6.44) from sample M54 of ACG. Visualization of the results about abundant and representative resistance genes was through a heatmap (Figure 1).

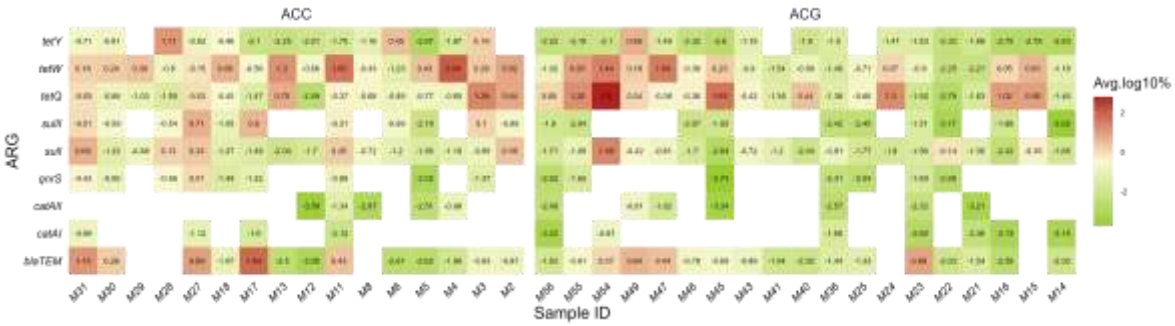

Figure 1. Antimicrobial resistance genes (ARGs) in fecal samples of coyotes (*C. latrans*) from Costa Rica collected from Central Conservation Area (ACC) and Guanacaste Conservation Area (ACG). The scale indicates the relative concentration of each gene. The white color represents the absence of ARGs.

In the ACC, high concentrations of *tet*W, and *tet*Q were observed around Irazu volcano, as long as sul1 gene was abundant in the eastern side of the Irazu. Nevertheless, the highest bla_{TEM} quantification was reported in Coronado (M17). In the case of ACG, *tet* concentrations were more abundant in the left side of the principal route in the sectors of the park, specifically in Santa Helena, Santa Rosa and Horizontes. Since *tet*Y and bla_{TEM} have been identified in the Pocosol M49 and M23, respectively (Figure 2).

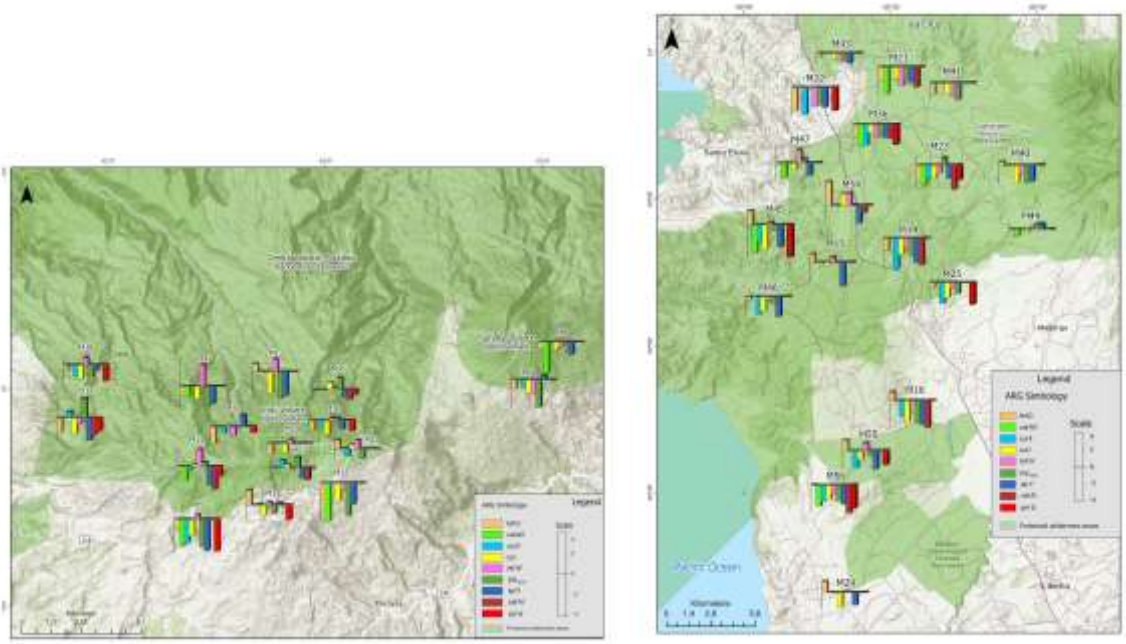

Figure 2. Maps of the conservation areas in Costa Rica showing land use and where scat samples of coyotes.

In the statistical analysis the principal component analysis of the heat map data determined that the *catA*II and *sul*II genes were the most important on axis one, while on axis two, the *tet*Q and *tet*Y genes were the most important (Table 3). The variance between component 1 and 2 was 84.56%.

Table 3. Eigenvalues of principal component (PC) analysis for antimicrobial resistance genes (ARGs) of coyotes (*C. latrans*) from Costa Rica. The genes with the highest weight are in bold.

Components 1 and 2 of the principal component analysis for resistance genes present in coyote feces from the Central and Guanacaste conservation areas of Costa Rica, years 2022.

Gen	PC1	PC2
bla$_{TEM}$	0.155140	-0.283977
*catA*I	-0.051282	-0.364123
*catA*II	**0.440568**	-0.013508

*qnr*S	0.439747	-0.057981
*sul*I	0.433581	-0.042573
*sul*II	**0.440954**	-0.013560
*tet*Q	0.045060	**-0.628346**
*tet*W	0.439847	-0.005182
*tet*Y	-0.088861	**-0.621599**

With the most important resistance genes, it was observed that there is a difference in the number of genes registered per conservation area and between the same genes (Permanova, F= 3.944; p= 0.0084). Thus, there is a difference between *sul*II, *tet*Q and *tet*Y in relation to *catA*II, the latter showing the lowest abundance. Meanwhile, *sul*II and *tet*Y genes have differences in their concentration between both conservation areas (Dunnpost huc, p< 0.05), which may indicate different types of contact with antibiotics Figure 3.

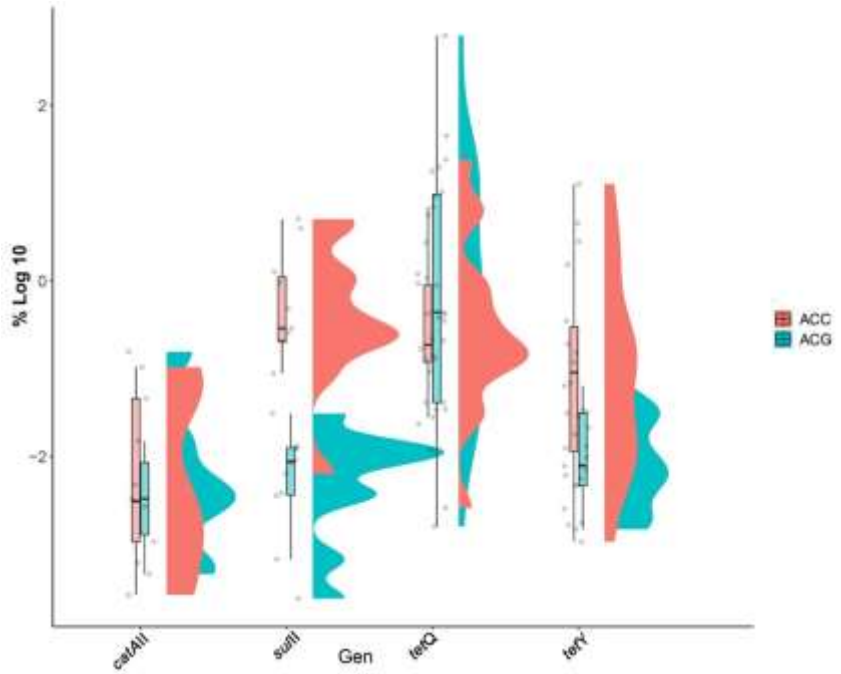

Figure 3. Differences between resistance genes from the heatmap in coyote feces from Central and Guanacaste conservation areas of Costa Rica, 2022. ACC: Central Conservation Area; ACG: Guanacaste Conservation Area.

As illustrated in Figure 4, the two primary canonical axes, which collectively account for 83% of the variance in the geography analysis, effectively describe the covert information associated with the relationship between ARGs. These relations will describe the relative transmission of ARGs to coyotes. The genes *tet*Y, *tet*W, *cat*AI and, *cat*AII, were found to be related to roads, while *sul*II was related to rivers. Additionally, bla_{TEM} was found to be related to livestock and pork production. Ultimately, the most representative genes of this analysis, bla_{TEM}, *tet*W and *tet*Q do not exhibit a significant divergence between groups or within each gene (Figure 5).

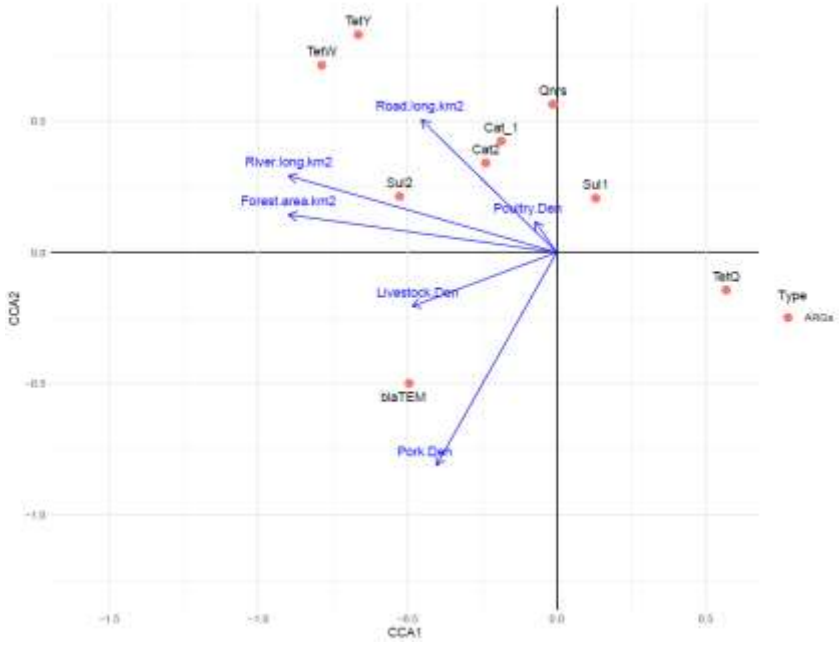

Figure 4. The canonical correspondence analysis for antimicrobial resistance genes in scat samples from coyotes (*Canis latrans*).

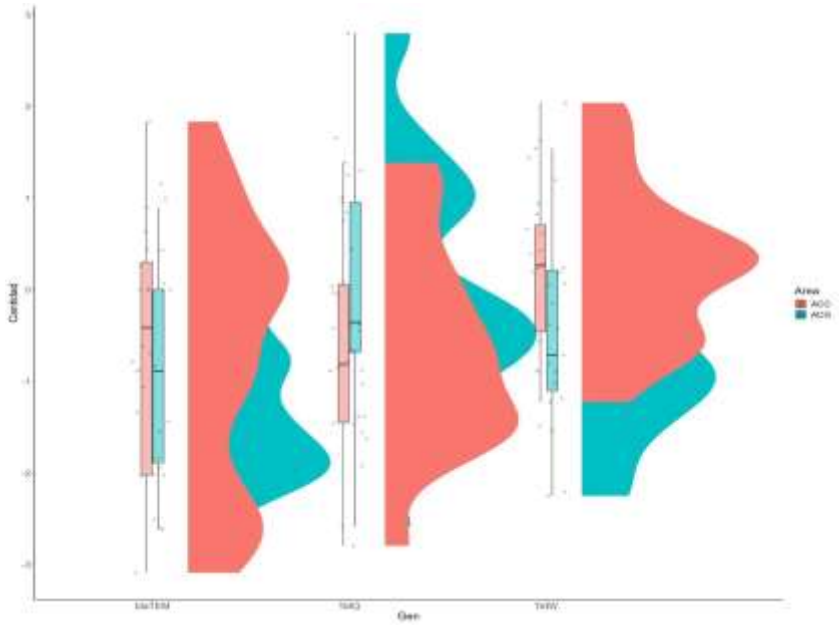

Figure 5. Differences between the most representative resistance genes from the canonical correspondence analysis in coyote feces from Central and Guanacaste conservation areas of Costa Rica, 2022. ACC: Central Conservation Area; ACG: Guanacaste Conservation Area.

Discussion

This article described the acquisition of ARGs by *C. latrans* in Costa Rica. The relevance of coyotes as bioindicators, it related with geographic and physical characteristics of the areas. Conditions to could demonstrate the source of acquisition of bacteria-containing ARGs. Monitoring a key species, as coyotes, gives information of the interaction of antibiotic resistance in the buffer areas between anthropogenic zones and reserve ecosystems. We evaluated 5 antibiotic classes through nine representative ARGs, highly used in human medicine and livestock: tetracyclines, sulfonamides, phenicols, quinolones, and betalactams.

The high presence of multidrug-resistant microbiome in the samples, could be associate to coyote characteristics of their opportunistic feeding habits. The behavior of search food traveling across various types of environments (rural and urban areas), given to them the acquire variety of microorganisms that carry on diversity of antimicrobial resistances genes (Awosile et al., 2023; Azofeifa-Romero et al., 2024). In fact, different studies support the high prevalence of multidrug-resistance microbial in wildlife. But our results are in

concordance to other coyotes antibiotic resistance analysis to confirm the multidrug-resistant microbiome in coyotes (Lee et al., 2022; Worsley-Tonks et al., 2020).

In Costa Rica, the percentage of ARGs encoding tetracyclines resistance is commonly the most frequent bacterial resistance in cases of human and domestic animals. Information related the same pattern in correlation with our study, where the three genes evaluated were representatives resistance genes in the statistics analysis (Mayorga et al., 2015).

*sul*II was the sulfonamide gene that exhibited differential amplification between the two areas and was related to the rivers according to the canonical correspondence analysis. This relationship coincides with the ecological differences, especially the annual rainfall between 2500 mm to 5500 mm in the Central Valley compared to Guanacaste (Valerio et al., 2004). Important feature because water has been recognized as an important vehicle of AMR but the public health impact attributed to the spread of AMR is poorly known (Domínguez et al., 2021).

Roads represent another source of distribution of ARGs in the environment. In our study we related association between the tetY, tetW, catAI and, catAII genes with roads. Data that are consistent with research findings on the spread of resistance genes. Finally, the gene bla_{TEM} was not the highest in amplifications but it was the most distributed in the samples analyzed, our study found association of this gene with livestock and swine production. The high amplifications found in Coronado of the Central Valley and in Pocosol of Guanacaste, agree with the land use reported for these areas and coincide with the studies of the frequent presence of bla_{TEM} in the wildlife interface and animal production systems.(Allen et al., 2010)

Conclusions

- The relative gene quantification data, based on the number of bacteria in each sample, revealed a significant difference in the number of genes registered per conservation area and between the same genes ($F= 3.944$; $p= 0.0084$). Specifically, *sul*II, *tet*Q, and *tet*Y exhibited a notable divergence from *cat*AII, which demonstrated the lowest abundance. The concentration of the *sul*II and *tet*Y genes differs between the two

conservation areas (Dunnpost huc, $p < 0.05$), which may indicate different types of contact with antibiotics.

- The application of spatial analysis facilitated an investigation into the correlation between the quantification of genes per sample and potential sources of contact. This revealed that the *cat*AI, *cat*AII, *tet*Y, and *tet*W genes are associated with roads, the *sul*II gene with rivers, and the bla_{TEM} gene with cattle and pig production systems.
- The coyote is confirmed as an effective sentinel species for monitoring the interface between human and natural environments, playing a crucial role in ecosystem health surveillance and in assessing the effectiveness of action plans and monitoring antibiotic resistance in Costa Rica.

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